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(54) Title: IMMUNOLOGICAL TOLERANCE TO HIV EPITOPES

(57) Abstract

Fusion immunoglobulin (fIg) proteins comprising one or more heterologous epitopes associated with a disease in which immune responsiveness is deleterious are useful to induce tolerance to these epitopes. HIV-1 gp120 epitopes linked in frame with an Ig heavy (H) chain are useful constructs for the induction of epitope-specific tolerance to HIV. Treatment of a subject with such a construct, or with lymphoid or hematopoietic cells expressing or secreting such fIg molecules induces specific immunological tolerance to those epitopes. Such tolerance, by preventing production of antibodies to selected gp120 epitopes, can prevent or inhibit "bystander" apoptosis of uninfected host T cells which have bound the HIV gp120 protein to their surface CD4 molecules and are subsequently cross-linked by undesired anti-gp120 antibodies, thereby priming them for apoptisis in the presence of antigens which activate those T cells. gp120 epitopes corresponding to non-neutralizing B cell epitopes or certain T helper cell epitopes are preferred for producing the fIg molecules. In addition to fIg H chains and complete Ig molecules, DNA encoding such H chain and cells transformed with such DNA are provided.

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IMMUNOLOGICAL TOLERANCE TO HIV EPITOPES

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This invention was funded in part by a grant from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, which provides to the United States government certain rights in this invention.

BACKGROUND OF THE INVENTION

Field of the Invention

The invention in the fields of immunology, molecular biology and medicine relates to compositions, primarily fusion immunoglobulins, and methods useful for inducing a state of immunological tolerance to selected epitopes of human immunodeficiency virus (HIV) gp120 or target epitopes assocaited with other diseases. Administration of these composition will induce and maintain tolerance to the epitopes in a subject infected with (or at high risk for) HIV, or in whom an immune response to a different target epitope is deleterious. Prevention of antibody responses to the selected HIV epitopes promotes survival of the host immune system and contributes to treatment of HIV disease. The compositions are also useful as adjuncts to HIV or other virus vaccines in modulating the immune response to maximize induction of protective anti-viral T cell immunity.

Description of the Background Art

Immunological tolerance (hereinafter "tolerance"), the basis of the lack of reactivity of the immune system to self components, can also be induced artificially by a wide variety of manipulations. Hence, an animals can be rendered tolerant to antigens which are foreign. Autoimmunity is thought to result in part from the breakdown of tolerance to previously tolerated antigens.

A variety of experimental procedures are known for inducing antigen-specific tolerance in neonates and adults (Billingham, R.E. *et al.* (1953) *Nature 172*:603-606; Chiller, J.M. *et al.* (1970) *Proc. Natl. Acad. Sci. USA. 65*:551-556; Borel, Y. *et al.* (1973) *Science 182*:76-78). In the immunocompetent adult, tolerance induction has been generally more difficult. Tolerance to foreign transplantation antigens or viral CTL epitopes, for example, was most effective in models where hematopoietic or

lymphoid ablation was followed by reconstitution with antigen-expressing bone marrow (BM)-derived antigen-presenting cells (APC) (Ildstad, S.T. *et al.* (1984) *Nature 307*:168-170; Cobbold, S.P. *et al.* (1984) *Nature 312*:548-551; Roberts, J.L. *et al.* (1990) *J. Exp. Med. 171*:935-940; Oehen, S.V., *et al.* (1994) *Cell. Immunol. 158*:342-352; Nemazee, D. *et al.* (1989) *Proc. Natl. Acad. Sci. USA 86*:8039-8043). For autoimmune diseases, studies have focused on the acquired induction of tolerance to autoantigens to prevent and/or ameliorate disease. For example, in murine models of multiple sclerosis or diabetes, prevention of disease has been accomplished with intrathymic, oral, or intravenous administration of , high doses of target autoantigens (Tisch, R. *et al.* (1993) *Nature 366*:72-75; Higgins, P.J. *et al.* (1988) *J.Immunol. 140*:440-445; Critchfield, J.M., *et al.* (1994) *Science 263*:1139-1143).

One well-known way to induce tolerance is by attaching the antigenic determinant or epitope to be tolerated to isologous or heterologous immunoglobulin (Ig) molecules, primarily of the IgG isotype. Such molecules are termed "tolerogenic carriers" or "tolerogens" (Scott, D.W. (1979) *Immunol. Rev. 43*:241). Igs of different origin may vary in their persistence in an animal after administration and/or in the mechanism by which they induce tolerance. However, IgG carriers have been by far the most efficacious inducers in adult animals of tolerance to haptens, nucleosides and peptides (Borel, Y. (1980) *Immunol. Rev. 50*:71; Scott, D.W. (1976) *Cell Immunol. 22*:311). These carriers owe their superior tolerogenicity to their persistence *in vivo* and the ability of an epitope chemically attached to the IgG molecule to crosslink membrane IgM (mIgM) on the surface of B lymphocytes with surface Fc receptors. However, chemical coupling of epitopes to IgG carriers can be limited by the availability of free reactive amino groups, structural change of the epitope as a result of the coupling reaction, and the uncontrolled targeting of the added determinant to different portions of the IgG.

Protein engineering strategies have been used to create molecules containing heterologous epitopes for the amplification of specific immune responses. For example, heterologous oligopeptide epitopes of immunological interest have been inserted in-frame into bacterial flagellin (Newton, S. *et al*, (1989) *Science 244*:70-72;

Jennings *et al.*, (1989) *Protein Eng. 2*:365), influenza virus nucleoprotein (Chimini, G. *et al.* (1989) *J. Exp. Med. 169*:97-302), hepatitis B surface antigen (Rutgers *et al.*, (1988) *Bio/Technology 6*:1065) and in the complementarity determining regions (CDR) of immunoglobulins (Billetta, R. *et al.*, (1991) *Proc. Natl. Acad. Sci. USA 88*:4713-4717; Zanetti *et al.* (1992) *Nature, 355*:476; Zanetti *et al.*WO90/090804); Zaghouani, H.*et al.* (1993) *Science 259*:224-227; Zaghouani, H. *et al.*, (1993) *Int. Rev. Immunol. 10*:265-278; Zaghouani, H. *et al.* (1995) *Proc. Natl. Acad. Sci. USA 92*: 631-635).

Attempts have been made to test the ability of such a recombinant protein to induce an enhanced immune response to the heterologous oligopeptide. A peptide immunoglobulin fusion Ig protein or referred to herein as a "fusion Ig" or "fIg" has been used to induce immunity. For example, a fIg was made which expressed in the CDR3 of its V_H region the repetitive tetrapeptide Asn-Ala-Asn-Pro (SEQ ID NO:1), designated $(NANP)_n$ (in single letter amino acid code), of the circumsporozoite protein of *Plasmodium falciparum*, an etiologic agent of malaria (Billetta et al., supra). A monoclonal antibody (mAb) specific for $(NANP)_n$ which was made against *P. falciparum* bound to the above fIg and was blocked by a synthetic $(NANP)_3$ peptide. Immunization of rabbits and mice with the engineered fIg in adjuvant elicited antibodies to the $(NANP)_3$ synthetic peptide and to *P. falciparum* parasite. Such antibodies efficiently inhibited the invasion of cultured liver cells by P. falciparum. Thus, immunity to malaria was induced in the absence of the parasite using antibody V regions engineered to mimic the parasite's molecular structure. The authors suggested that antibody (idiotype) mimicry of an exogenous antigen is possible and may only require a discrete stretch of identity for successful mimicry. An alternate and simpler explanation of these results by the present inventors is that this material, when administered in adjuvant, simply acted as an immunogenic hapten-carrier conjugate. C. Bona *et al.*, (1994) *Cell Mol. Biol. 40 Suppl 1*:21-30 expressed viral epitopes on Ig molecules by replacing the D segment of a V_H gene with a B cell epitope from the V3-loop of HIV-1 envelope glycoprotein gp120, a cytotoxic T lymphocyte (CTL)-epitope from influenza virus nucleoprotein or a T helper epitope

from influenza hemagglutinin. The T cell-targeted peptides in the form of fIg molecules produced by cells transfected with chimeric V genes, activated specific T cells. The authors speculated about possible practical applications for Ig molecules bearing foreign epitopes for the development of prophylactic and immunotherapeutic reagents.

It is noteworthy that Zanetti *et al. (supra)* and Bona *et al. (supra)* produced chimeric Ig molecules (which are fIgs as the term is used herein) for the purpose of immunization (vaccination), not tolerization. Although WO90/09084 casually proferred a speculative notion, lacking any particularity or evidence, that this type of construct could be used for tolerization, the authors provided no scientific basis for such a utility. In fact, the way in which their exogenous epitope was inserted into the Ig framework region resulted only in immunogenic, not tolerogenic, constructs. The Zanetti *et al.* reference therefore lacks any proof that its inventors were in possession of a tolerogenic fIg and provides no enabling support for any tolerogenic molecule or preparation. Hence, the induction and maintenance of tolerance to oligopeptides presented to the immune system via engineered Ig proteins has not been demonstrated prior to the invention as described herein. In particular, the art has not seen the use of cells (expressing such fIg molecules) as agents of epitope-specific tolerance induction or maintenance. The present invention is the first discovery of tolerogenic cellular engineering to achieve a meaningful effect with therapeutic utility.

In summary, the art recognizes that recombinant fusion proteins, including fIg proteins, may be useful as immunogens to induce immune responses to the heterologous oligopeptide. However, there remains a recognized need to develop general and specific methods of inducing stable, long-lasting tolerance to any of a number of epitopes of clinical significance in a subject. Also needed are vectors that can introduce the target epitope to which tolerance is desired into a host cell or whole animal, such that the epitope (a) induces tolerance and (b) persists *in vivo* so that it maintains the tolerant state. It is essential that any tolerization protocol include a means to maintain the specific state of tolerance. Maintenance of tolerance is understood to require the persistence of the tolerogenic epitope *in vivo* (Smith, R.T.

(1961) *Adv. Immunol. 1*:67; Golub, E.S. *et al.* (1967) *J. Immunol. 99*:6; Ramsdell, F. *et al.* (1992) *Science 257*:1130-1134).

The present inventors were the first to discover an approach that not only could induce tolerance to an oligopeptide presented to the immune system in the form of a recombinant fIg protein but also could maintain a tolerant state in the subject. See also Scott and Zambidis, co-pending, commonly assigned application U.S.S.N. 08/195,874 (allowed) and WO95/21926, which applications are hereby expressly incorporated by reference in their entirety.

One of the present inventors' central hypotheses for explaining the signalling process in tolerance is that crosslinking with anti-μ chain antibodies provides "signal 1" to B cells, which, in the absence of T cell help (signal 2), leads to anergy. At high concentrations of anti-μ, extensive crosslinking of IgM leads to a significant level of B-cell apoptosis because a greater proportion of the B cells are forced to exit the G_0 phase and enter the cell cycle. This effect can be mimicked by multivalent antigen in specific B cells (Carsetti, R. *et al.*, (1993) *Eur. J. Immunol. 23*:168). A unifying explanation for various experimental results is that that multiple crosslinking events are necessary for the induction of apoptosis. (See, also, Warner, G. *et al.* (1991) *Cell. Immunol.*, 138:404; Scott, D.W. *et al.* (1987) *Immunol. Today, 8*:105; Alés-Martínez, J.-E. *et al.* (1992) *Sem. in Immunol. 4*:195; Scott *et al.* (1996) *Intern. Immunol. 9*:1375-1385).

The Immune Response to HIV gp120 and its Role in AIDS

The immune response to HIV has been studied extensively. Early studies suggested a role for neutralizing antibodies in protection or containment of HIV infection. This is particularly true in the case of simian immunodeficiency virus (SIV), a relative of HIV, where a cloned virus could be employed (Burns, D. *et al.* (1993) *J. Virol.* 67:4104). Neutralizing antibodies to the epitopes of the envelope glycoprotein gp120, especially the V3 loop, have been described in infected individuals. However, more recent evidence suggests that the antibody response to HIV may not be protective and may, in fact, contribute to the progression of disease (Füst, G. *et al.* (1995) *Immunol. Today, 16*:167; Wang, S. *et al.* (1994) *Virology*

199:247; Banda, N. *et al.* (1992) *J. Exp. Med. 176*:1099). Thus, while antibody responses against various epitopes of HIV clearly occur, the effectiveness and therapeutic significance of these responses is unclear.

The CD4 molecule on T lymphocyte serves as receptor for major histocompatibility complex (MHC) class II antigens and is referred to as "coreceptor" because its engagement synergizes with engagement of the T cell receptor for antigen (TCR) in activating the cells. When CD4 molecules were engaged by antibody independently of the TCR (in murine studies), the T cells were induced to undergo apoptosis (Wang, Z.Q. *et al.* (1994) *Eur. J. Immunol. 24*:1549-1552). Thus, besides functioning as a coreceptor with the TCR, CD4 has a function of its own in facilitating the induction of apoptosis. CD4 also serves as a cellular binding site or receptor for the HIV gp120. In transgenic mice expressing a human CD4 transgene, appropriate crosslinking of gp120 caused massive deletion of HIV-reactive T cells *in vivo* (Wang, Z.Q. *et al.* (1994) *Europ. J. Immunol. 24*:1553-1557). If T cells in which CD4 is engaged by anti-CD4 antibody administration are capable of expressing functional Fas protein on their surface, they degrade their DNA and disintegrate rapidly.

Antibodies to gp120 can lead to enhancement of HIV entry into non-T cells via Fc receptors (Homsy, J. *et al.* (1989) *Science* 244:1357, *supra*). Uptake of complexes between HIV and anti gp120 antibody by cells of the immune system, particularly monocytes, can result in establishment of a latent, subclinical infection and a virus reservoir susceptible to later activation(Kliks, S.C., (1993) *Proc. Natl. Acad. Sci. USA 90*:11518)). HIV-infected patient sera frequently contain antibodies against a peptide of the gp120 C5 region which cross-react with HLA-C monomorphic determinants (DeSantis, C. *et al.* (1993) *J. Infec. Dis. 168*:1396; Palker, T.J. *et al.* (1987) *Proc. Nat'l Acad. Sci. USA 84*:2479). Though apparently not causing autoimmune damage, the antibodies are an example of non-protective antibodies produced during HIV disease. In an equine retroviral disease model, an antibody response to a variant virus (EIAV) may end in more extensive disease (Cook, R. *et al.* (1995) J. *Virology 69*:1493). Moreover, production of non-

neutralizing anti-HIV antibodies (specific for "irrelevant" or "ineffective" epitopes) may pre-empt the formation of antibodies to important, neutralizing epitopes.

In AIDS, shifts in cytokines from those produced by T_H2 cells to cytokines made by T_H1 cells were observed (Clerici *et al.* (1994) *Proc. Natl. Acad. Sci. USA 91*:11811; Gougeon, M-L. *et al.* (1993) *Science.* 268:1269; Ameisen, J-C. (1992) *Immunol. Today. 13*:388). While controversial (Fauci, A. (1993) *Science* 262:1011), such shifts may contribute both to apoptosis and to hypergammaglobulinemia. The findings discussed above have led those developing the next generation of vaccines to re-direct the immune response in patients and to devise T-cell peptides that serve as stimulatory ("vaccine") and target epitopes for cytotoxic T lymphocytes (Salk, J. *et al.* (1993) *Science* 260:1270; Cease, K.B *et al.* (1994) *Ann. Rev. Immunology. 12*:923.

Evidence obtained in the last few years suggests that HIV may subvert the immune response through the interaction of viral gp120 with the CD4 receptor on T cells. Observations from Finkel's laboratory (Finkel *et al.*, *supra*; Banda *et al.*, *supra*) and of Newell *et al.* (1990) *Nature 347*:286), indicate that crosslinking of CD4 on the T cell surface may prime T cells for apoptosis, perhaps via the upregulation of the Fas molecule, CD95 (Oyaizu, N. *et al.* (1994) *Blood 84*:2622; Desbarats, J. *et al.* (1996) *Proc. Natl. Acad. Sci. USA 93*:11014-11018. Even picomolar concentrations of gp120 could prime T cells for such activation-induced death.

Apoptosis in normal, non-infected ("bystander") $CD4^+$ T cells may be programmed by (1) allowing gp120 proteins to bind to CD4 via their natural affinity, and then (2) adding anti-gp120 antibodies to bind and crosslink the gp120-CD4 complexes (Finkel *et al.*, *supra*; Banda *et al.*, *supra*). When such programmed or "primed" cells are triggered through their TCR, apoptosis follows. This is reminiscent of the increased rate of apoptosis observed *in vitro* in T cells from HIV-infected subjects (Gougeon, M-L. *et al.* (1993) *Science.* 268:1269; Ameisen, *supra*) and provides one explanation for CD4+ T cell depletion in AIDS: According to this view, concurrent infection by other organisms or any antigenic challenge for that matter would trigger the death of those T cells which bear a TCR recognizing these antigens and in which the CD4 molecules have been crosslinked via gp120 and anti-

gp120. Such a mechanism may also explain (1) the enhancement of infection brought about by certain anti-HIV antibodies, and (2) the paradox that HIV appears to cause AIDS after the onset of antiviral immunity.

Ex vivo analysis of $CD4^+$ T cells (without prior culture) indicates that the increased apoptosis representes a process ongoing *in vivo*. Varying numbers of $CD4^+$ as well as $CD8^+$ cells in lymph nodes (LN) of HIV infected subjects are undergoing apoptosis. In infected adults and children, a fall in the CD4/CD8 ratio correlates with increasing apoptosis of $CD4^+$ cells which correlates with CD4 depletion and disease severity. In infected humans and monkeys, most apoptosis in the LNs was occurring in "bystander" (uninfected) cells. Among infected cells, those expressing lower levels of the HIV p24 protein showed higher levels of apoptosis than cells expressing higher levels of p24. Thus, the majority of apoptosis appears to takes place in HIV^- cells, and the majority of apoptotic cells are HIV^- or HIV^{low}.

The mechanism for such T cell apoptosis has been suggested by Pahwa and colleagues (Oyaizu, N. *et al.* (1993) *Blood 82*:3392-3400) who examined apoptosis as a mechanism for CD4+ T cell depletion in HIV-1 infection. They showed that
(1) patient blood mononuclear cells underwent marked spontaneous apoptosis;
(2) stimulation of patient and normal T cells resulted in increased apoptosis; and
(3) cross-linking of CD4 molecules was sufficient to induce apoptosis in CD4+ T cells if cross-linking was performed in unfractionated blood mononuclear cells (but not in purified T cells). The accelerated cell death through apoptosis was concluded to play an important role in the pathogenesis of HIV-1 infection, and crosslinking of CD4 *in vivo* contributed to this mechanism. Cross-linking of CD4 molecules, induced either by anti-CD4 monoclonal antibody (mAb) or by HIV-1 envelope protein gp160 (which includes gp120) upregulates Fas mRNA and Fas antigen expression in normal lymphocytes (Oyaizu *et al.* (1994) *surpra*). Upregulation of Fas antigen closely correlated with apoptotic cell death. CD4 cross-linking resulted in the induction of interferon-γ (IFNγ) and tumor necrosis factor-α (TNF-α) in blood cells, both of which cytokines contributed to Fas upregulation. Anti-IFN-γ and anti-TNF-α antibodies blocked crosslinking-induced Fas upregulation and lymphocyte apoptosis. Hence,

aberrant cytokine secretion induced by the crosslinking of CD4 and the consequent upregulation of Fas antigen were concluded to play a critical role in triggering peripheral T cell apoptosis and thereby contribute to HIV disease pathogenesis. Cross-linking of the CD3 molecule (present on all T cells) caused an increase in the Fas ligand. This combination of increased expression of Fas and Fas ligand led to apoptosis.

Based on the foregoing, the present inventors have concluded that the antibody response to gp120 in an infected subject is an important pathway leading to AIDS progression due to the pathogenetic component of $CD4^+$ T cell depletion through bystander apoptosis as described above. Therefore, they have developed novel compositions and methods based on their general, flexible approach to the induction and maintenance of epitope-specific tolerance to eliminate virus-specific immune responsiveness. In particular T helper cell and/or antibody responsiveness to one or more epitopes of viral gp120 is prevented or inhibited through the induction and maintenance of immune tolerance in T helper cells, B cells or both that are specific for one or a number of selected HIV gp120 epitopes.

Furthermore, the present inventors have extended this approach to the induction of tolerance to any antigen, be it an autoantigen, an antigen of a microorganism or a tumor antigen, against which an undesired antibody response or T helper cell response occurs in a disease setting and is pathogenic or otherwise deleterious to the host.

Citation of the above documents is not intended as an admission that any of the foregoing is pertinent prior art. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicant and does not constitute any admission as to the correctness of the dates or contents of these documents.

SUMMARY OF THE INVENTION

The present inventors have devised novel fusion proteins and DNA constructs coding therefor. The fusion protein includes a desired peptide epitope or several epitopes, toward which immune tolerance is to be established, inserted in particular

sites of the immunoglobulin ("Ig") heavy ("H") chain. This product is termed a "fusion immunoglobulin" and is abbreviated "fIg" herein. A preferred fIg includes an epitope or epitopes of HIV-1, most preferably from the gp120 glycoprotein of HIV-1. To make this construct, DNA encoding the targeted epitope or epitopes is inserted "in frame" within a DNA construct encoding the Ig heavy (H) chain. If two or more targeted epitopes are included, they exist as contiguous or non-contiguous sequences in the protein from which they are derived, and may be either linear or conformational epitopes.

This fusion protein construct is then transfected into a cell line, preferably a myeloma or other line of B lymphocyte lineage (such as a human cell line transformed by Epstein-Barr virus) that produces Ig light (L) chains but that cannot produce H chains due either to a spontaneous or induced mutation. When the transfected Ig H chains are synthesized, they combine naturally with the host cell's Ig L chains to form complete immunoglobulin molecules (H_2L_2) which are secreted. This resultant Ig fusion protein contains the desired target epitope (or epitopes) preferably in its N-terminal region and functions as a tolerogen for both B cells and T cells and induces tolerance *in vivo*. Transgenic mice producing such a fusion protein are highly tolerant immunologically to the epitopes included in the fIg. The present inventors have found that Ig fusion proteins such as these can be presented to the immune system in a tolerogenic fashion, either as an fIg preparation or in the form of transgenic hemopoietic precursor cells or B cells expressing the fIg, to induce both B and T cell tolerance to the targeted HIV-1 gp120 epitope..

The present inventors have conceived of an approach that is useful in producing improved and effective immunity against a virus, in particular, human immunodeficiency virus (HIV-1, HIV-2) by inducing tolerance to selected nonprotective viral epitopes as discussed above. Thought a peptide comprising the desired epitopes can be chemically attached to an autologous Ig carrier for tolerance induction, the present inventors have created a fIg comprising one or more peptide epitopes and the Ig H chain using recombinant methods as described herein.

The invention specifically involves:

1. engineered synthesis of peptide immunoglobulin fusion proteins that are highly tolerogenic.
2. cells transduced with DNA encoding such an engineered fIg to induce, but more importantly, to maintain, a state of tolerance to the peptide epitopes.

The invention provides polynucleotides encoding the fIg in the form of recombinant DNA molecules in vehicles such as plasmid and retroviral vectors, capable of expression in a desired eukaryotic host cell as disclosed herein. The invention also provides hosts transfected or transduced with the fIg constructs which are capable of producing in culture or *in vivo* the fIg molecules and secreting them or displaying them on the cell surface.

This invention is useful for the treatment of any disease in which immunologic reactions are pathologic. The best-known examples are in infectious and autoimmune diseases. In many types of infections, where the host response to the organism damages the host. For example in certain arenavirus infections (for example, lymphocyte choriomeningitis virus infection), the T cell response is responsible for as much or more pathology than the virus itself. Antibody responses and the interaction of the antibodies with complement is responsible for the hemorrhagic shock syndrome elicited by flaviviruses (in particular) dengue virus or arenaviruses, such as Junin virus which causes Argentinean hemorrhagic fever. In both the latter cases, an efficient immune response leads to disaster for the host. Other examples of diseases for which the present invention can be used include viral diseases wherein virus-antibody complexes damage the host. For example, infants congenitally infected with cytomegalovirus have such circulating complexes that are deposited in the kidney. Patients with hepatitis B virus infection have circulating complexes that result in arthritis and glomerulonephritis. Antibodies generated against a virus can also act as autoantibodies directed against normal tissues, even tissues not infected directly by the virus. An example of this is the polyendocrinopathies that develop in newborn animals infected with reovirus type 1 in whom antibodies against antigens in pancreatic islets, the anterior pituitary and the gastric mucosa have been observed.

Such examples abound in the art and may be found in any comprehensive treatise on microbiology or infectious diseases.

More classical autoimmune diseases are also treatable by the present invention as either cell-mediated or antibody responses to organ-specific antigens or common or cross-reactive antigens are the known pathogenic agents. Specific tolerance induced by an fIg of this invention is a promising therapeutic approach to the treatment of many types of autoimmune disease.

The invention provides an individual fIg H chain or fIg H chain dimers. Also provided by the invention is an fIg molecule comprising (i) two different H chains, one of which is a fusion protein having one or more HIV gp120 epitopes included in the V region, preferably at the N-terminus of a framework region, most preferably of the first framework region, and (ii) native L chains. Preferably, both H chains of the fIg molecule are the fused H chains.

Specifically, the present invention is directed to a fusion immunoglobulin (fIg) heavy (H) chain protein comprising a mammalian, preferably human, Ig H chain fused in frame after the leader in its N-terminal region to one or more HIV gp120 epitopes, wherein the fIg H chain is tolerogenic in a host with respect to the gp120 epitopes. The tolerogenic epitope(s) is or are fused to the variable region of the Ig H chain, preferably at the N terminus of a framework region of the variable region. Most preferably the HIV gp120 epitope or epitopes are fused to the N-terminal amino acid residue of the mammalian Ig H chain such that all amino acids encoding the gp120 epitope or epitopes are N-terminal to the Ig-encoding amino acids.

Also provided is an intact fIg protein comprising two Ig H chains and two Ig L chains, wherein at least one of the H chains is the fIg H chain described above. Preferably, both of the H chains are the above fIg H chains. A preferred Ig is one which fixes complement and has a longer serum half life. Thus, in a preferred embodiment, the fIg H chain is an Ig γ chain, more preferably an Ig γ_1, γ_2 or γ_3 chain. Most preferably, the Ig is human IgG and preferred fIg isotypes are IgG_1, IgG_2 and IgG_3.

In one embodiment of the above fIg, the one or more gp120 epitopes comprises a full length gp120 protein In other embodiments, the gp120 epitopes are one or more peptides selected from the group consisting of the C1 region, the V3 loop and the C5 region.

In yet other preferred embodiments, the gp120 epitope is a B cell epitope comprising a peptide selected from the group consisting of: VPVWKEATTTLFCASDAKAY (SEQ ID NO:2), EVHNVWATHACVPTD (SEQ ID NO:3), YDTEVHNVWA (SEQ ID NO:4), PQEVVLVNVT (SEQ ID NO:5), PQEVVLVNVTENFDMWKNDM (SEQ ID NO:6), PNNNTRKSIR (SEQ ID NO:7), NNNTRKRIRIQRGPGR (SEQ ID NO:8), RKSIR (SEQ ID NO:9), IQRGPGRAFV (SEQ ID NO:10), GRAFVTIGKI (SEQ ID NO:11), PGRAFY (SEQ ID NO:12), NTRKSIRIQRGPGRAFVTIG (SEQ ID NO:13), PNNNTRKSIRIQRGPGRAFVTIGKIGNMRQAHC (SEQ ID NO:14), NNTRKSIRIQRG (SEQ ID NO:15), NKRKRIHIGPGRAFYTTKNIIGTIC (SEQ ID NO:16), RKSIRIQRGPGRAFV (SEQ ID NO:17), IRIQRGPGR (SEQ ID NO:18), KRIRIQRGPGRAFVTIG (SEQ ID NO:19), QRGPGRAF (SEQ ID NO:20), RGPGRAFV (SEQ ID NO:21), RKRIHIGPGRAFYTT (SEQ ID NO:22), RGPGRAFVTIG (SEQ ID NO:23), SISGPGRAFYTG (SEQ ID NO:24), KRIHI (SEQ ID NO:25), KRIHIGP (SEQ ID NO:26), IHIGPGR (SEQ ID NO:27), HIGPGR (SEQ ID NO:28), HIGPGRA (SEQ ID NO:29), HIGP (SEQ ID NO:30), RIHIGPGRAFYTTG (SEQ ID NO:31), RIQRGPGRAF (SEQ ID NO:32), IQRGPGRAFV (SEQ ID NO:10), IQRGPGRAF (SEQ ID NO:33), IRIQRGPGRAFVTI (SEQ ID NO:34), RGPGRAFVTIGKIG (SEQ ID NO:35), QRGPGRA (SEQ ID NO:36), IXXGPGRA (SEQ ID NO:37), IGPGR (SEQ ID NO:38), GPGR (SEQ ID NO:39), GPXR (SEQ ID NO:40), GPGRAF (SEQ ID NO:41), RIHIG (SEQ ID NO:42), HIGPGRAF (SEQ ID NO:43), GRAF (SEQ ID NO:44), GGGDMRDNWRSELYKYKVVK (SEQ ID NO:45), KYKVVKIEPLGVAPTKAKRR (SEQ ID NO:46), LGVAPTKAKR (SEQ ID NO:47), GGDMRDNWRSELYKYKVVKI (SEQ ID NO:48), IEPLGVAPTK (SEQ ID NO:49), RRVVQRE (SEQ ID NO:50), PTKAKRR (SEQ ID NO:51) and VVQREKR (SEQ ID NO:52).

In yet other preferred embodiments, the gp120 epitope is a T helper cell epitope comprising a peptide selected from the group consisting of: EQLWVTVYYGVPV (SEQ ID NO:53), VYYGVPVWKEA (SEQ ID NO:54),

GVPVWKEATTLFC (SEQ ID NO:55), AHKVWATHACV (SEQ ID NO:56), NVWATHACVPTD (SEQ ID NO:57), CVPTNPVPQEVV (SEQ ID NO:58), VEQMHEDIISLW (SEQ ID NO:59), EQMHEDIISLWDQ (SEQ ID NO:60), EQMHEDIISLWDQSL (SEQ ID NO:61), HEDIISLWDQSLK (SEQ ID NO:62), VTVYYGVPVWKEATTTLFC (SEQ ID NO:63), VVLVNVTENFNM (SEQ ID NO:64), SLKPCVKLTPLCY (SEQ ID NO:65), CTRPNNNTRKSIRIQRGPG(Y) (SEQ ID NO:66), NTRKSIRIQRGPGR (SEQ ID NO:67), EQRGPGRAFVTIGKI (SEQ ID NO:68), RIQRGPGRAFVTIGK (SEQ ID NO:69), RIHIGPGRAFYTTKN (SEQ ID NO:70), GRAFVTIGKIGNMRQ (SEQ ID NO:71), QRGPGRAFVTIGKIGNMRQAH (SEQ ID NO:72), VGKAMYAPPISGQIR (SEQ ID NO:73), GNSNNESEIFRPGGG (SEQ ID NO:74), FRPGGGDMRDNWRSEL (SEQ ID NO:75), DMRDNWRSELYKYKV (SEQ ID NO:76), RDNWRSELYKYKVVK (SEQ ID NO:77), CKYKVVKIEPLGVAPT (SEQ ID NO:78), YKYKVVKIEPLGVAP (SEQ ID NO:79), KVVKIEPLGVAPTKAKRRVVQREKRC (SEQ ID NO:80), ITLPCRIKQIINMWQEVGKAMYAPPISGQIRC (SEQ ID NO:81), and ELYKYKVVKIEPLGVAPTKAKRRVVQREKR. (SEQ ID NO:82)

The present invention is further directed to a DNA molecule comprising a nucleotide sequence encoding any fusion Ig H chain as described above.

Also provided is an expression vector useful for producing the above fusion Ig product and for inducing and maintaining immunological tolerance to one or more epitopes of HIV gp120 protein in a subject, preferably a human. The vector preferably comprises (a) a DNA molecule as above, operably linked to (b) transcriptional and translational control regions operable in a hematopoietic cell or lymphoid cell of the subject. The transcriptional and translational control regions provide for constitutive expression of the DNA sequence in a lymphoid cell or a hematopoietic cell. A preferred vector is a retroviral vector. A naked DNA vector may also be used.

The present invention also provides a hemopoietic or lymphoid cell transformed by a vector as above, which cell stably expresses the flg protein. Stable expression is expression which is not transient, and persists for weeks or even months, preferably for the *in vivo* lifespan of the cell in which the flg is expressed. Such a cell is preferably a human bone marrow cell, a resting B

lymphocyte or an activated B lymphocyte which has been activated by a mitogen or other polyclonal B cell activator.

Also included is a method for producing the fusion Ig of the invetnion by culturing the above transformed cell. For *in vitro* production of the fIg, however, any cell type may be used which can express an Ig H chain gene as well as the DNA encoding the fIg and secrete it into the culture medium.

The present invention includes a pharmaceutical composition comprising:

(a) a tolerogenic amount of a fusion Ig molecule having a fusion Ig H chain as described above; and

(b) a pharmaceutically acceptable carrier or excipient for parenteral administration.

Preferably, in the pharmaceutical composition, the fIg is an isologous IgG molecule.

Also provided herein is a method for immunologically tolerizing a subject to one or more HIV gp120 epitopes comprising administering to the subject an effective amount of a fusion Ig pharmaceutical composition as described above.

A method for immunologically tolerizing a subject to one or more HIV gp120 epitopes comprising introducing into the subject an effective amount of transformed cells as described above, thereby tolerizing the subject.

In another embodiment, the invention is directed to a method for immunologically tolerizing a subject to one or more HIV gp120 epitopes comprising introducing into the subject an effective amount of transformed cells as above, thereby tolerizing the subject. Prior to introducing the transformed hemopoietic cells into the subject, the subject may be treated to diminish the host's hemopoietic cells, although this may not be necessary in a patient with AIDS. Tolerance may also be achieved by a combination of treatment with transformed cells and a pharmaceutical composition comprising fIg as described above.

This invention is also directed to a method of (i) inducing and (ii) maintaining immunological tolerance to an epitope or epitopes of HIV gp120 protein in a subject, comprising:

(a) administering to the subject an effective amount of a fIg pharmaceutical composition as described above to induce the tolerance to the epitope or epitopes; and

(b) administering to the subject an effective amount of transformed hemopoietic or lymphoid cells as described above to maintain the tolerance to the epitope or epitopes,

thereby inducing and maintaining the tolerance. However, tolerance is also induced and maintained by means of administering the transformed hemopoetic or lymphoid cells without resorting to the fIg itself. Thus, expression of the fIg by the transformed cells is sufficient to accomplish the induction and the maintaining functions.

Also included is a method for identifying whether a candidate HIV gp120 epitope or epitopes are tolerogenic in a first subject when presented to the subject in a fusion Ig molecule, comprising the steps of:

(a) producing a expression vector as above, wherein the gp120 epitope or epitopes in the fusion Ig are the candidate epitopes;

(b) stably transforming a population of autologous or matched allogeneic cells of the subject with the vector;

(c) introducing the transformed cells into the subject; and

(d) determining whether the subject is tolerant to the candidate epitope or epitopes by

(i) immunizing the subject with the candidate epitope or epitopes in immunogenic form and measuring the immune response *in vivo* or *in vitro*, and

(ii) comparing the response to an immune response in a second control subject similarly immunized which has not been treated with the transformed cells,

such that, if the first subject is tolerant, the candidate epitope or epitopes in the fusion immunoglobulin construct is identified as being tolerogenic. In the above method, the subjects are preferably humans and the transformed cells are human cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 (sheets 1/1 through 1/3) shows the amino acid sequence of HIV-1 gp120 (mature protein). The boldface sequence, SEQ ID NO:83, (with position numbers in the right margin) is the consensus sequence of the protein from subtype B virus (the prevalent HIV-1 subtype in the United States). The following characters are used in the consensus sequence:

(1) single letter code; UPPER CASE letter indicates that the amino acid residue in that position is conserved for all known viral isolates of subtype B;

(2) lower case letter indicates the amino acid residue is conserved in more than 50% of known isolates;

(3) a "?" indicates lack of consensus at that position (no single residue is found in the majority of isolates).

The concensus sequence is read left to right. Shown **vertically** below each position in the consensus sequence (where appropriate) are alternative amino residues that have been identified at that position in mutants or variants of subtype B. Residues which happen to be adjacent to one another below the consensus sequence line are NOT to be read left to right as they do **NOT** represent adjacent residues in an actual gp120 sequence. (Note: all the variant residues below the consensus sequence line are UPPER CASE for clarity only.) All of the sequence information in Fig. 1 (and Fig. 2) was obtained from *The Human Retroviruses and AIDS Genetic Sequence 1995 Compendium*, published by the Los Alamos National Laboratory: Theoretical Biology and Biophysics Division, Los Alamos, NM.

Figure 2 (sheets 2/1 - 2/4) shows the aligned consensus sequences for the major subtypes or "clades" of HIV-1 as published in *The Human Retroviruses and AIDS Genetic Sequence 1995 Compendium* (see Figure 1). HIV subtypes are defined and distinguished based on their nucleotide (and not amino acid) sequences. Certain

"signature sequences" are characteristic of a subtype, for example, the GPGR consensus sequence at the tip of the V3 loop of the subtype B that appears as a GPGQ consensus for most other subtypes. The letter/symbol conventions are the same as those used in the consensus sequence in Figure 1. In addition, the presence of a "." at a position indicates that most isolates (and hence, by definition, the consensus sequence for the subtype) lack an amino acid at that position. All sequences are shown relative to the consensus A sequence (SEQ ID NO:84), and a "-"indicates the same residue as in subtype A at that position. The gp120 sequences in Figure 2 (SEQ ID NOS:84-104) include the signal peptide (N terminal to the mature protein and indicated above the CONSENSUS-A sequence). The mature protein begins to the right of the "/" Other landmarks indicated include the V3 neutralization loop, the C terminus of gp120 (indicated by a "/" on sheet 2/4) and the N-terminal segment (about 16 residues) of the HIV-1 gp41 protein.

Figure 3 (sheets 3/1 and 3/2) shows the aligned amino acid sequences of gp120 (including the signal sequence) from several strains or isolates of HIV-1. The top line of each grouping (in boldface) is the subtype B consensus sequence (SEQ ID NO:105; also appearing in Figures 1 and 2). The footnotes describing each variant or isolate and the markings used in Figure 3 are as follows:

(1) The first 27 - 30 amino acids left of the "//" mark comprise the signal sequence of gp120. The mature gp120 protein begins to the right of the "//". In general, a space appears after each 10 residues. To preserve alignment, spaces have sometimes been omitted and for the consensus sequence, additional residues have been placed above the main sequence line.

(2) CON-B is the consensus sequence for gp120 of subtype B (SEQ ID NO:105). UPPER or lower case letters are as described for Figs. 1 and 2. The presence of single letter amino acid codes or "?" above the consensus sequence line indicates the existence of additional residues in some subtype B isolates at approximately the positions indicated. In some locations, arrows appear in the sequence line as place indicators for such additional residues. Each arrow is not intended to correspond to a single residue and points to the known residues (usually "?") that may occupy that region in various isolates..

(3) **BH10** isolate (SEQ ID NO:106): Ratner, L. *et al. Nature 313*:277-284(1985) (Genbank SWISS PROT Accession No. P03375)

(4) **LAV-BRU** isolate (SEQ ID NO:107): Wain-Hobson, S. *et al., Cell 40*:9-17(1985) (Genbank SWISS PROT Accession No. P03377)

(5) **ARV2/SF2** isolate (SEQ ID NO:108): Sanchez-Pescador, R., *et al., Science 227*:484-492(1985) (Genbank SWISS PROT Accession No. P03378)

(6) **MN** isolate (SEQ ID NO:109): Gurgo, C. *et al.*, *Virology 164*:531-536(1988) (Genbank SWISS PROT Accession No. P05877)

(7) **92US712.4** isolate (SEQ ID NO:110). This sample was part of a set of sequences generated through the NIAID/NIH DAIDS HIV variation program. The virus was derived from an asymptomatic individual from Baltimore, thought to be infected by parenteral i.v. drug user contact. The *env* sequence clustered with HIV-1 B subtype sequences. Gao, F. *et al., J. Virol. 70*:1651-1667 (1996) (Genbank SWISS PROT Accession Number U08449). This sequence was randomly chosen as a subtype B isolate for illustrative purposes and for comparison with the more common variant sequences.

Figures 4A and 4B illustrate a preferred engineering strategy for inserting a foreign epitope at the N-terminus of an IgG γ chain. Figure 4A depicts the incorporation of an oligonucleotide (SEQ ID NO:111) encoding the λ phage C1 repressor peptide 12-26 (SEQ ID NO:112) as described in Examples. This fIg was expressed in murine J558 myeloma cells. The present invention introduces an oligonucleotide or polynucleotide encoding one or more native or synthetic gp120 peptide epitopes into an Ig H chain, preferably a human γ chain (Figure 4B).

Figures 5A and 5B show strategy for the construction, expression, and epitope recognition of a fusion Ig gene by inserting a foreign epitope into a V_H gene. Fig. 5A presents a scheme for constructing the fIg. A modified 12-26 nucleotide sequence was ligated into a *Pst*I site of a 1.3-kb murine V_H (LVDJ) chain fragment. The *Pst*I site appears at the coding sequence of the fifth amino acid of the FR1; therefore, a repeat of the first five FR1 amino acids was designed to follow the coding sequence of the 15 amino acids of 12-26, so as not to perturb proper framework region folding after insertion. SDS/10% polyacrylamide gel electrophoresis of purified H-chain transfected immunoglobulins demonstrated proper assembly of H chains with L chains. Fig. 5B is a gel pattern showing recognition of epitopes by immunoblotting. Purified control IgG (P6) of 12-26-IgG (Q3) samples were electrophoresed on SDS/10% polyacrylamide gels, transferred onto nitrocellulose, and probed with anti-mouse IgG1 (left lanes) or with biotinylated anti-12-26 mAb B3.11 (right lanes) plus AP-conjugated secondary reagents.

Figure 6 shows *in vivo* effects of 12-26-IgG pretreatment on peptide-specific humoral immune responses. Male BALB/c mice were injected i.v. with a single 1-mg

dose of deaggregated protein G-purified P6 (▲) or Q3 (/) IgG. Mice were immunized and total or isotypic anti-peptide IgG titers were quantitated by ELISA 8 days after a secondary antigenic boost. Isotyped anti-peptide titrations (IgG_1 and IgG_{2b}) represent mean absorption values from assays of three individual mice in each group. 0 , Preimmunization sera

Figure 7 shows *in vivo* effects of 12-26-IgG pretreatment on peptide-specific cellular immune responses. Tertiary cytokine (IL-2 and IL-4) responses of enriched splenic T cells (3×10^6 cells per ml) from mice displaying tolerized humoral immune secondary responses are shown. IL-2 and IL-4 production in supernatants was determined in triplicate by CTLL and CT.4S assay, respectively. "Medium only" backgrounds were subtracted; these values ranged from 1 to 4 units/ml in all assays.

Figure 8 shows structure and genomic Southern blotting of transgenic mice expressing 12-26-IgG specifically in the B-lymphocyte lineage. A murine IgG_1^b H chain construct containing endogenous immunoglobulin promoter and enhancer (E_H) sequences was modified to express 12-26 peptide and a repeat of perturbed framework region sequence (FR1) at the N-terminus. Fertilized embryos were injected with this linearized construct and transgenic mice were generated via standard procedures. Genomic DNA from tail biopsies was digested with *Bam*HI and *Eco*RI to release a 1.3 kb V_H fragment, fractionated on 0.8% agarose/TBE, and transferred onto nylon membranes via alkaline Southern transfer . Southern blots were probed with random-primed ^{32}P-labeled DNA sequence containing 3 tandem repeats of 12-26 nucleotide sequence. Densitometry studies using known amounts of purified, linearized transgene DNA was used to estimate that there are 2-3 integrated copies in Lines 5 and 17.

Figures 9A and 9B show profound peptide-specific cellular and humoral immune tolerance in 12-26-IgG-expressing transgenic mouse lines. Fig. 9A presents titers of total anti-peptide IgG (open symbols), or IgG_1 isotype (closed symbols) for Line 5 transgenic mice measured after peptide immunizations and secondary boosts. Fig. 9B presents splenic T cell cytokine responses from tolerant Line 5 trangenic (Tg)

mice determined by CTLL assay. Error bars signify the standard error of the mean for 3-4 mice per group.

Figure 10 shows profound peptide-specific cellular and humoral immune tolerance in transgenic bone marrow chimeras expressing 12-26-IgG. Chimeras were prepared with 1:1 mixtures of Line 17 Tg and non-transgenic (NTg) bone marrow (/). Antibody responses to peptide are shown. Anti-HEL specificity controls showed no differences between groups. Nonirradiated mice injected with saline (0) displayed immune responses similar to control chimeras reconstituted with 100% NTg bone marrow (◇). Error bars signify standard error of the mean of 2-3 mice per group.

Figure 11 shows the induction of peptide-specific humoral immune tolerance in normal immunocompetent adults by intravenous injection of various preparations of 12-26-IgG-expressing lymphoid tissues. Normal, nonirradiated BALB/c males were injected iv with $4x10^7$ sex-matched splenocytes, Percoll®-gradient-purified (60-70% fraction) resting B cells, 48-hour activated LPS blasts, or crude unfractionated bone marrow cells from Line 17 transgenic mice. Recipients were rested for 7-10 days before immunization with 50 μg peptide in CFA (SC base of tail). Mice were boosted with an additional 50 μg in saline 2 weeks later and serum antibody titers determined 8 days later.

Figures 12A and 12B present an analysis of B-cell tolerance induction in tolerized transgenic or normal adult subjects. Fig. 12A: Nontransgenic (◇), Line 5 transgenic (∇), or line 17 transgenic (/) mice were immunized intraperitoneally with 50 μg 12-26-HEL conjugate in CFA, and boosted with the same in saline 2 weeks later. Anti-peptide and anti-HEL (all $>10^5$, not shown) titers were determined by ELISA as described in the text. Fig. 12B: Serum titers from adoptively transferred recipients boosted with 50 μg 12-26-HEL conjugate in IFA were similarly determined: BALB/c recipients were irradiated with 400 rads, and injected with 5 x 10^7 splenocytes from Line 17 Tg-tolerized donors (closed circles, various sources of lymphoid tissue) or non-transgenic injected, non-tolerized donors (open diamonds). Splenic donors had been previously primed and boosted with 12-26 peptide and HEL

(at different subcutaneous locations), and had previously displayed tolerance (experiment from Figure 11).

Figures 13A. 13B,and 13C summarize studies showing the induction of tolerance in previously-primed adult recipients by either resting, B cells, B cell blasts or chemically fixed B cells. BALB/c mice were immunized SC with 50 μg 12-26 peptide in CFA 1-2 weeks before iv injection of 4x107 Line 17 transgenic (!) or nontransgenic control (◇) purified resting B cells (Fig. 13A), LPS-activated B cell blasts (Fig. 13B), or carbodiimide-fixed B cells (Fig. 13C). The mice were challenged IP with 50 μg soluble peptide 1-2 weeks following tolerizing injections, and antibody titers (ELISA) determined 8 days later. The graphs show peptide-specific total IgG or two IgG isotypes (IgG_1 and IgG_{2b}),

Figures 14 and 15 show B cell expression, epitope recognition, and direct antigenic presentation of retrovirally-synthesized peptide-IgG. Fig. 14 shows the structure and proviral integration of murine Moloney leukemia retroviral construct MBAE.BAK. Ten μg of genomic DNA from transduced, G418-resistant (+) or control (-) A20 cells was digested with Sac I, fractionated on 0.8% agarose, Southern-blotted, and probed with ^{32}P-labeled DNA probe containing three tandem copies of 12-26 sequence. Sac I digestion releases an ~5.1 kb proviral genome. Fig. 15 shows tissue expression of 12-26 mRNA in long-term (~3 months) recipients of gene-transferred (✚) or mock-transduced (—) BM progenitors. RNA from bone marrow (B), thymus (T), or spleen (S) was assayed by 12-26 sequence RT-PCR (25). One-tenth of each PCR reaction (except for A20 controls: 1/100th) was Southern-blotted and probed with a non-complementary ^{32}P-labeled 12-26-specific oligonucleotide.

Figure 16 shows the induction of peptide-specific cellular immune tolerance in adult bone marrow chimeras infused with peptide-Ig-expressing progenitor cells. BALB/c mice were sublethally irradiated (600 rads) and injected iv with $1\text{-}2x10^6$ gene-modified or mock-transduced BM. Recipients were immunized with peptide in CFA 2 months post-infusion and draining LN cells were restimulated *in vitro* with dilutions of synthetic peptide and 25-50 μg/ml purified protein derivative (PPD,

Connaught) in RPMI 1640 with 0.5% heat-inactivated autologous serum. Stimulation indices (SI) represent ratios of proliferation to medium alone backgrounds (5,609±2,271 cpm). IL-2 and IFN-γ were quantitated by CTLL and ELISA assays, respectively (Gilbert, K.M., *et al.* (1994) *J. Exp. Med. 179*:249-258). Additional experiments also revealed a diminution of peptide-specific IL-4 release in LN of tolerized recipients. Error bars signify standard error of the mean for 3 individual mice per group. This experiment was done at least twice with 3-4 mice per group with similar results.

Figures 17A, 17B and 17C show the induction of peptide-specific humoral immune tolerance in adult bone marrow chimeras infused with peptide-Ig-expressing progenitor cells. BALB/c mice were sublethally irradiated with either (A) 200 rads (Fig. 17A) , or 600 rads (Fig. 17B,C) and infused with 1-2x10^6 gene-transferred (triangles) or mock-transduced (circles) BM cells. Mice were primed and boosted for humoral responses either (Fig. 17A) one month, or (Fig. 17B,C) 2 months post-infusion with synthetic 12-26 peptide, and HEL as a specificity control. Non-manipulated, immunized BALB/c always produced titers similar to recipients infused with mock-transduced BM cells (Fig. 17A, diamonds). Both total peptide-specific IgG (open symbols), or the main isotype IgG1 (closed symbols) were diminished in all experiments. Normal recipients in Fig. 19B and 19C received either 5-FU-pretreated normal BALB/c BM or SCID/BALB/c BM cells. Flow cytometric analysis at the one month sacrifice time (of mice from Fig. 17A) revealed comparable levels of CD4+ and Ig+ splenocytes in normal BALB/c reconstituted with either normal or SCID BM: (CD4+: 18-25%; Ig+: 40-65%). All experiments were done at least twice with 3-5 mice per group with similar results.

Figures 18-19 show peripheral tolerance induction in immunocompetent adults with gene-transferred peripheral B cells expressing engineered peptide-Ig. Figure 18 shows humoral and cellular tolerance induction. Unirradiated mice were injected with >1x10^7 LPS blasts co-cultured with retrovirus-producing F6P (+) or mock-transducing ψ-2 (-). One week later, mice were primed and boosted for humoral responses, and sacrificed 3 months later for analysis of splenic memory T cell

responses. Cytokine release in individual splenic cultures was determined at 24 hours (IL-2) or 48 hours (IL-4); medium alone background values were less than 1-2 U/ml and were subtracted for clarity (ΔU/ml). Figure 19 shows persistence of gene-transferred B cells. Hybridomas generated from spleens of tolerized mice by PEG fusion of A20 cells with LPS-activated splenocytes (48 hours, 50 μg/ml LPS). Hybridomas were selected in 1 mg/ml G418 and tested for their ability to activate T-cell hybrid 9C127 as above. Eight representative A20 hybridomas from each recipient (Mice #1-3) are shown.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The nucleotide and amino acid sequences of most known isolates of HIV-1 are published in "The Human Retroviruses and AIDS Genetic Sequence 1995 Compendium" (hereinafter, "Compendium"), as well as earlier editions of the Compendium). This document is available in paper or electronic form from its publisher, the Los Alamos National Laboratory: Theoretical Biology and Biophysics Division, Los Alamos, NM. The complete nucleotide and amino acid sequences of individual HIV isolates as well as consensus sequences for recognized HIV-1 subtypes or clades (A, B, C, D, E, F, G, O U, CPZ) have been published in this database for a number of years. The *HIV Molecular Immunology Database 1995*, Editors: B. Korber *et al.*, Los Alamos National Laboratory: Theoretical Biology and Biophysics, Los Alamos, NM, 1995, (referred to herein as the "HIVMID") provides T-cell epitope maps, alignments, and annotation (for T helpe epitopes and for CTL epitopes) , as well as a summary and map of linear B cell epitopes and monoclonal antibodies recognizing such epitopes. This application incorporates by reference the latest Compendium and HIVMID, but is also intended to include updates containing sequences of additional viral isolates as they are added and published. The compendium (database) and HIVMID are publicly available on the World Wide Web at the address "http://hiv-web.lanl.gov".

In particular, the HIV-1 *env* gene (which encodes the gp160 precursor protein of both gp120 and gp41 envelope proteins) consensus nucleotide sequences for 10

viral subtypes appear at pages I-A-358 to I-A-364 of the 1995 Compendium (NOV 1995). The *env* nucleotide sequences of individual viral isolates, grouped by subtypes and appearing along with the consensus sequence for the subtype, are at pages I-A-174 to I-A-357 of the 1995 compendium.

Crosslinking of CD4 molecules on human T cells either by (a) HIV-1 virions bound to CD4 via viral gp120, or (b) anti-gp120 antibodies crosslinking of soluble gp120 bound to CD4, primes or programs the T cells for apoptosis, as described herein. Thus, an infected subject's antibody response to HIV-1, particularly to one or more epitopes of gp120, contributes to the pathogenetic process by targeting bystander T cells to self-destruct. The inventors have discovered an approach to modulate these responses by inducing selective immunological tolerance either at the level of B cells, T helper cells or both, resulting in diminished antibody responses to one or more gp120 epitopes. Inducing and maintaining such tolerance in a subject provides a therapeutic approach for treating HIV infection. Furthermore, such tolerance induction to selected epitopes can be used as part of a therapeutic or prophylactic vaccine approach. Thus, an improved HIV vaccine may include in addition to an HIV immunogenic preparation, a flg in accordance with this invention to reduce or prevent undesired antibodies.

The term "tolerant" or "tolerance" as used herein is defined functionally in terms of the immune response to an immunogenic challenge with an antigen. A subject is tolerant if his response to an immunogenic challenge is reduced by at least about 50%, more preferably at least about 80% relative to a non-tolerant control subject.

Tolerance may be manifest by reduced reactivity *in vivo* such as antibody formation or *in vitro,* for example, by reduced lymphocyte proliferation.

A "tolerogen" is a form of antigen which, when it encounters the immune system, induces a state of immunological tolerance or hyporesponsiveness or anergy in the host. Such a state is tested by subsequent immunization or challenge of lymphocytes *in vitro* with the specific antigen in immunogenic form.

The term "immunogenic" with reference to an antigen or epitope is also a functional term which is dependent on the nature, form, dose and route of administration of the antigen (epitope) such that it has immunogenic properties, *i.e.*, it induces immune reactivity resulting in antibodies or cellular immunity. Thus the same molecule, e.g., a protein, can be immunogenic or tolerogenic depending on it form (e.g., aggregated or deaggregated) dose or route of administration, all of which is well-known in the art. As described herein, antigens, including low molecular weight haptens, can be rendered non-immunogenic and even tolerogenic by coupling them to homologous immunoglobulin molecules. In fact, a key observation underlying this invention is that such "coupling" can be achieved by recombinant techniques in the form of a fIg wherein a peptide epitope (or "antigenic determinant" or minimal antigenic structure) for which tolerance is desired is made part of the fIg using methods described herein.

Because certain tolerogenic fIg preparations, or cells expressing such an fIg, can induce hyporesponsiveness in an already primed or immunized subject (see below), the present invention is useful as a therapeutic tolerogen, to curtail an ongoing immune response to a selected gp120 epitope or epitopes during the course of HIV disease. In fact, that may be the more significant clinical utility of this invention. Alternatively, the fIg tolerogen is to modulate the response to an HIV vaccine such that the subject immunized with the vaccine and treated with the tolerogen responds to particular desired viral epitopes (expressed by the vaccine) and is prevented (or suppressed) in his response to other selected epitopes (expressed by the tolerogen) By judicious selection of these HIV epitopes, most preferably gp120 epitopes, it is possible to render a host selectively tolerant at the level of T helper cells, B cells, or both. In the case of HIV infection, this would inhibit or prevent the production of antibodies that are of no benefit (*e.g.*, non-neutralizing), and more importantly, are harmful via mechanisms such as bystander apoptosis or enhancing antibodies which promote infection of host cells. On its face, it might appear counterintuitive to inhibit an immune response to a virus which one wishes to eradicate. However, given the differences between epitopes of HIV recognized by antibodies and by cytotoxic T

lymphocytes (CTL) (see HIVMID). B cell and/or T helper cell tolerance to one or more (even all) epitopes of gp120 molecule may still permit an effective CTL response against other (non-tolerizing) gp120 epitopes or, importantly, other non-envelope HIV proteins which are known to be immunogenic. HIV T helper epitopes and CTL epitopes have been described in a number of publication, for example, Berzofsky, J.A. (1995) *Ann N Y Acad Sci, 754*:161-168; Meister GE *et al.* (1995) *Vaccine, 13*:581-591; Cease KB *et al.* (1994) *Annu Rev Immunol, 12*:923-989; Shirai M; *et al.* (1994) *J Immunol, 152*:549-556; Ahlers JD *et al.* (1993) *J Immunol, 150* 5647-5665; Berzofsky JA, (1991) *Biotechnol Ther, 2*:123-135; De Groot AS, *et al.* (1991) *J Infect Dis, 164*:1058-1065; Berzofsky JA *et al.* (1991) *J Clin Invest, 88*:876-884; Clerici M *et al.* (1991) *Eur J Immunol, 21*:1345-1349 Palker TJ *et al.* (1989) *J Immunol, 142*:3612-3619, which references are hereby incorporated by reference.

The present inventors have developed a flexible fusion protein approach for induction of unresponsiveness to defined B-cell and T-cell epitopes *in vivo* and *in vitro.* See, for example, Scott and Zambidis, co-pending application U.S.S.N. 08/195,874, PCT Publication WO 95/21926 and Zambidis, E.T. *et al.*, (1996) *Proc. Natl. Acad. Sci. USA 93*:5019-5024, which references are hereby incorporated by reference in their entirety. As described herein, this approach originally set forth for other antigens, is adapted for the production of compositions and methods useful for inducing unresponsiveness to one or more HIV gp120 epitopes.

Epitope-specific tolerance is used to ablate undesired antibody responses while maintaining protective CTL responses. Hence, by inducing B cell tolerance and T helper cell tolerance to all gp120 epitopes, either by use of a fIg into which a complete gp120 sequence or one or more partial gp120 sequences have been inserted, or by using a mixture of fusion Ig's each including a subset of gp120 epitopes, anti-gp120 antibody responsiveness can be prevented or diminished. Because the CD8 arm of the immune response is not affected, protective antiviral cell-mediated immunity, in particular CTL responses to HIV epitopes, remains intact.

IgG-gp120 peptide fusion proteins are effective tolerogens which modulate anti-gp120 responses. Furthermore, human hematopoietic precursor cells, whether from BM or other tissues, and their progeny B cells which express the IgG-gp120 peptide fusion proteins are themselves tolerogenic agents which deliver or present on their surface the selected HIV peptides in tolerogenic form for induction and/or maintenance of the tolerant state. Thus, in one embodiment, the ongoing maintenance of tolerance is achieved by first transfecting bone marrow (BM) cells or peripheral hematopoietic stem cells from any tissue (for example, $CD34^+$ peripheral blood stem cells in the human) with a DNA vector which includes a DNA sequence encoding a IgG-gp120 fusion protein of the present invention. In another embodiment, the tolerogen is presented expressed in a myeloid cell (as determined in studies using SCID mouse BM). The B cell expressing the tolerogenic fIg may be a resting B cell, an activated B cell or B cell blast, or a transformed B cell (*e.g.*, leukemia or lymphoma) which has been appropriately attenuated to ablate its oncogenic potential for use in human subjects. Long-lasting, even permanent tolerance can be induced by grafting transfected BM stem cells or peripheral stem cells. This approach is described in more detail in Example IV.

Tolerogen Presentation

B cells are known to be capable of inducing tolerance by presentation of appropriate surface molecules in a tolerogenic fashion (Eynon, E.E. *et al.* (1992) *J. Exp. Med. 175*:131, using human IgM and IgD; Fuchs, E. *et al.* (1992) *Science 258*:1156, for the H-Y antigen).

The present inventors discovered that resting B cells expressing a fIg, after injection into a recipient subject, induce tolerance for natural epitope included in the fIg, such as the phage λ 12-26 epitope. Larger blast cells induced by stimulating such B cells with bacterial lipopolysaccharide (LPS) (termed "LPS blasts") also tolerize for this peptide. Activated B cells are better tolerogenic vehicles in primed recipients than resting B cells. This is in contrast to the observations of Yuschenkoff *et al.* (*supra*) who found that activated B cells from mice transgenic for and expressing human μ chains lost the ability to tolerize. Transgenic lymphoma cells activated in

this way did not induce tolerance but rather appeared to induce an immune response for the same epitope.

In murine studies, splenic B cells stimulated with LPS are infected with a retrovirus construct containing the desired epitope. In a model system, the 12-26 IgG flg has been used successfully in this way. Such LPS blasts are tolerogenic for that epitope. Hybridomas produced from the splenic B cells expressing the flg also express the flg transgene. When transgenic BM expressing 12-26 flg or normal (control) BM is injected into recipient mice irradiated with 200R, and the animals are immunized with the peptide in immunogenic form (in adjuvant), the following results have been obtained:

(1) T cells in recipients of transgenic BM are tolerant, measured by T cell proliferation and production of cytokines (IL2, IL4, IFN-γ,. *etc.*).

(2) recipients are tolerant as far as making IgG antibodies to the peptide.

Tolerance to a desired HIV gp120 peptide epitope included in an flg construct is achieved using as a source of B cells expressing the flg on their surface any population of lymphocytes known to contain B cells or to differentiate into B cells. This may include an unfractionated population, a cell preparation enriched in B cells or their precursors, or a purified B cell population. Any conventional method for enriching or purifying B cells may be employed. Examples of tissue sources for B cells include BM, spleen, LN, peripheral blood or lymph. B cells may be resting or preferably are activated, for example, LPS blasts.

As described in the Examples, when normal murine spleen cells were first stimulated by LPS followed by infection with a retroviral vector carrying the flg transgene and then infused into normal recipients, followed by immunization, the following results were obtained: The T cell responsiveness to the 12-26 peptide showed decreased IL-2 and IL-4 production. Animals had a decreased antibody response to the peptide. The effect on the antibody response can be explained by the T helper cell compartment being tolerant, the B cells being tolerant, or more likely, both. Table I summarizes results in a primed subject:

TABLE I

Recipient	Outcome Measure	Small B Cells	LPS Blasts	Fixed B Cells[1]
Normal	IgG Titers	↓↓	↓↓	ND[2]
Primed[3]	IgG1 Response	= not tolerogenic	↓↓	partial ↓

[1] Fixed cells are treated with a carbodiimide.
[2] ND=not determined
[3] Recipients injected 10 days earlier with 12-26 in complete Freund's adjuvant.

Without wishing to be bound by any mechanism, the present inventors propose two possible mechanisms to explain these results:

(1) Antigen presentation without "signal 2" (R. Schwartz (1989) *Cell 57*:1073-1081) results in anergy.

(2) LPS blasts may induced "propriocidal" cell death..

Lenardo and colleagues (Boehme S.A. *et al.* (1993) *Eur. J. Immunol. 23*:1552-1560; Boehme S.A. *et al.* (1993) *Leukemia 7 (Suppl 2)*:S45-S49; Critchfield, J.M. *et al.* (1995) *Cell. Immunol. 160*:71-78; Pelfrey, C.M. *et al.* (1995) *J. Immunol. 154*:6191-6202) found that stimulated T cells (or T cell hybridomas) produced IL-2 but also underwent suicide termed "propriocidal death." This response is thought to be important for regulating an ongoing immune response wherein suicide of responding cells serves to bring the response to a timely termination.

Evaluation of Potentially Tolerogenic gp120 Epitopes for Use in fIg

Human γ globulin (HGG) (American Red Cross), a model tolerogenic carrier, is used as a carrier in these evaluations of a given peptide ("PEP") corresponding to one or a combination of epitopes of gp120. MBS (*m*-maleimodobenzoyl-N-hydroxysuccinimide ester) is a preferred coupling agent because of ease of use and thiol-cleavability (*i.e.,* to prepare control peptide-conjugates, as well as to determine conjugation ratios). A known antigen (hapten) may be used as a specificity control for tolerance, *e.g.,* FITC-coupled HGG. In a standard protocol based upon long-term experience in the present inventors' laboratory (Scott, D.W. *et al.* (1979) *Immunol.*

Rev. 43:241; Warner, G.L. *et al.* (1991) *J. Immunol.* 146:2185), murine spleen cells are cultured for 24 hours with increasing concentrations of PEP-HGG, FITC-HGG or anti-μ (positive control for tolerance); these cells are washed and then challenged with LPS in microculture for 4 days. ELISAs for IgM and IgG anti-PEP, anti-gp120, anti-HGG and anti-FITC are then performed by standard methodology. This protocol allows for polyclonal stimulation that elicits measurable responses to all of these epitopes Once established, the evaluation can be performed in PEP-primed subjects to verify that tolerance induction can be achieved in secondary B cells (Linton PJ, *et al.* (1991) *J. Immunol.* 146:4099).

It is also helpful to perform dose response studies using PEP-HGG conjugates, as well as free peptide, administered intravenously. For example, groups of 4-5 mice are injected intravenously with 0.1, 0.3 or 1 mg of PEP alone, PEP-HGG, or FITC-HGG as a specificity control. Four to seven days later, mice are challenged with gp120 in complete Freund's adjuvant (CFA). Mice are bled on day -7 (before tolerance) and at 10 and 20 days after challenge; mice can then be boosted on day 20 and bled 7 days later to evaluate secondary IgG responsiveness. Heterologous IgG's are known to be tolerogenic *in vivo* at $<10^{-8}$ M (~0.1-1 mg/mouse). Peptides for inducing T cell tolerance are commonly administered at higher concentrations (approximately 10^{-7}M).

It may also be advantageous to establish epitope density requirements for tolerance. Typically, hapten-protein ratios of 5-10 are used with Ig conjugates. It would be desirable to control coupling reactions to achieve molar ratios (PEP:HGG) of 2,4,8, and 16. Because the MBS cross-linker is cleavable, it is possible to quantitate ratios and create peptide-linker only controls. Primed recipients may require tolerogens with a higher epitope density. In the fIg embodiment, higher epitope density is translated into inclusion of more copies of the DNA encoding the epitope, for example 2-10 copies, in the fIg DNA construct if this is required to overcome a state of preexisting immunity in the subject.

Achievement of tolerance using the above chemical-coupling approaches along with determinations of optimal dose-response relationships and epitope

densities will provide the basis and indicate the efficacy of the epitopes to be inserted into the recombinant fIg fusion proteins for use as tolerogens.

Choice of HIV gp120 Epitopes for B Cell and T Helper Cell Tolerance Induction

The tolerogenic IgG-gp-120 peptide fusion proteins may include one or more peptides of gp120, including the full-length gp120 protein. If more than one peptide epitope is present, the different peptides may be arranged in the fusion protein in the same order and in contiguous form as they are in the native gp120 protein. Alternatively, the peptides may be "reshuffled" in the fusion protein. Furthermore, one or more of the gp120 peptides may be present in the fusion protein in two or more copies, either alone or with another gp120 peptide. In a preferred embodiment, the one or more epitopes selected for use in the tolerogenic fIg is a linear epitope. However, as conformational epitopes become better defined, it will be possible to construct a fIg having one or more epitopes which, in combination, yield the conformational determinant in the expressed fIg.

It is advantageous to use the largest fragment of the native gp120 protein that (a) can be fused with the Ig H chain while maintaining the required tertiary structure of the Ig portion of the fusion protein for tolerogenic activity and (b) can be accommodated by the vector used to transfer the fIg-encoding DNA. The advantage lies in the fact that the appropriate epitopes of such a fIg are selected by the host MHC proteins (of antigen-presenting cells or, in this case, tolerogen-presenting cells) for presentation and tolerance induction. In humans, this would obviate the need to select *a priori* those epitopes of gp120 which would interact with the HLA-DR molecules of a given subject to yield an active tolerogen for that subject. As more information relating various HL-A types with HIV gp120 epitopes becomes available, it will become easier to tailor smaller tolerogenic epitopes for a given subject. Approaches to accomplish this for T helper cells epitopes are already available through various computer based algorithms which are discussed in much more detail below.

Expression of the epitope on the fIg can be tested using a conventional immunoassay with an antibody specific for the epitope (if it is a B cell epitope) or with lymphocyte proliferation or cytokine secretion assay (for a T helper cell epitope).

Antibodies recognizing such epitopes are available, and T cells can be prepared *in vitro* or long-term T cells lines of the appropriate specificity are available or can be prepared using conventional methods. The Compendium, and in particular the HIVMID lists antibodies specific for each of the epitopes of Table II, for example. The antibodies may be rodent mAbs, human polyclonal or mAbs or hybrid antibodies generated from such human or rodent mAbs. Alternatively or additionally, the soluble flg can be administered in adjuvant to a host and tested for generation of peptide-specific T-cell responses *in vivo*, due to processing and presentation by endogenous APC, even in the context of an Ig scaffold (see Examples).

A gp120 epitope of the present invention, in particular a linear or "sequential" epitope, is preferably one comprising a "natural" sequence, defined as the sequence as it occurs in a consensus gp120 sequence of a particular HIV subtype or a naturally occurring mutant thereof which has been isolated and characterized.

However, the epitope sequence may also be a variant of a natural sequence defined here as a sequence in which one or more amino acid residues has been replaced by a different residue, including substitutions not known to occur in natural viral isolates. The only condition is that the variant sequence maintain the secondary and tertiary structure needed to create the desired the tolerogenic epitope when expressed in a flg protein either in solution or on a cell surface. Hence, it is preferred that any variant maintain (a) the structure of the peptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the substitution site, or (c) the bulk of the side chain.

For a detailed description of protein chemistry and structure, see Schulz, G.E. *et al., Principles of Protein Structure,* Springer-Verlag, New York, 1978, and Creighton, T.E., *Proteins*: *Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, 1983, which are hereby incorporated by reference. The types of substitutions which may be made in the gp120 protein or peptide molecule of the present invention may be based on analysis of the frequencies of amino acid changes between a homologous protein of different species (*e.g.,* Table 1-2 of Schulz *et al.*

(*supra*) and Figure 3-9 of Creighton (*supra*). Base on such analysis, conservative substitutions are defined as exchanges within one of the following five groups:

1. Small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr (Pro, Gly);
2. Polar, negatively charged residues and their amides: Asp, Asn, Glu, Gln;
3. Polar, positively charged residues: His, Arg, Lys;
4. Large aliphatic, nonpolar residues: Met, Leu, Ile, Val (Cys); and
5. Large aromatic residues: Phe, Tyr, Trp.

The three amino acid residues in parentheses above have special roles in protein architecture. Gly is the only residue lacking a side chain and imparts flexibility to the chain. Pro, because of its unusual geometry, tightly constrains the chain. Cys participates in disulfide bond formation which is important in protein folding. Tyr, because of its hydrogen bonding potential, has some kinship with Ser, Thr, *etc.*

Most deletions and insertions, and substitutions according to the present invention are those which do not produce radical changes in the structural or immunological characteristics of the gp120 protein or peptide molecule when expressed as part of a fIg. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays. For example, a variant typically is made by chemical synthesis or site-specific mutagenesis of the peptide-encoding nucleic acid, expression of the variant nucleic acid in an fIg construct in recombinant cell culture, and, optionally, purification from the cell culture, for example, by immunoaffinity chromatography using an immobilized antibody specific for the natural (non-variant) epitope. The presence of the desired epitope can be readily ascertained by one skilled in the art using an antibody, for example in an immunoassay using a mAb the binding of which defines the epitope. A standard assay, for example immunofluorescence of flow cytometry may be used to detect the variant epitope on the surface of a cell. Alternatively, the presence of the desired epitope can be detected using a cellular assay, for example an assay which measures the stimulation of T lymphocytes to proliferate or to secrete

cytokines. Such assays are well known in the art and are described in detail in the Examples below.

In preferred embodiments, a tolerogenic gp120-IgG fusion Ig protein modulates the responsiveness of B and/or T cells to non-neutralizing gp120 epitopes, for example in the C1 region or in the C5 region which contains HLA-cross-reactive C5 epitopes.

The C1 region of gp120 is noteworthy for its dominance in being a target for immune reactivity. The V3 loop, in particular the V3 region of the loop (see Tables II and III), is noteworthy as a target for neutralizing antibodies. The C5 region is noteworthy for its cross reactivity with HL-A molecules and its stimulation of autoimmunity. Hence one or more epitopes of one of more of these regions would be useful as tolerogenic epitopes. In particular any gp120 epitope which stimulates autoimmunity or against which an autoimmune host response is directed (irrespective of mechanism of induction) is a preferred epitope for use in a tolerogenic flg of this invention.

Because the host immune system will process any administered flg for presentation to T lymphocytes in conjunction with the host's MHC glycoproteins on the antigen-presenting cells ("APC")(or more appropriately, "tolerogen-presenting" cells), it is preferably to include a peptide of sufficient length for binding to host MHC molecules and subsequent presentation. As shown below, in particular for T helper cell epitopes, the gp120 peptide may be as short as about 6 amino acids. Generally larger peptides are preferred, including those with more than one gp120 epitope in the flg. For example, about 10-20 amino acids, preferably about 10-40 amino acids, more preferably about 10-60 amino acids are included in the flg. This will allow the host cells to select the epitopes appropriate for the particular MHC type. Alternatively, as discussed below, T helper epitopes may be identified and selected using various published computer-based algorithms. It is preferable, though not required, to exclude cysteine from the tolerogenic flg because of the constraints this amino acid imposes on uncontrolled secondary structure.

The present inventors have developed a model system which utilizes mice made transgenic for human CD4 which is used to screen fIg constructs for their efficacy and utility in humans. Administration to these mice of gp120 and anti-gp120 or HIV virions or crosslinked gp120 leads to sensitization for subsequent apoptosis. Once the animals have been primed in this way, apoptosis occurs "spontaneously" in response to environmental exposure to antigens which engage the TCR and trigger the apoptotic process. Specific antigens including peptides with defined epitopes are administered to more precisely activate T cell apoptosis. This can be evaluated by testing the animals for epitope-specific T cell unresponsiveness or hyporesponsiveness. The induction of B-cell tolerance and T helper cell tolerance to selected gp120 epitopes can readily be tested in this model for its effect on the pathway of T-cell apoptosis.

The present inventors have utilized a peptide that contains both a T-cell and a B-cell epitope, created a fusion protein of this peptide with an IgG molecule serving as a "carrier" and have used it to induce epitope-specific T-cell and B-cell tolerance (see Examples). This success indicates the utility of inducing epitope-specific B-cell tolerance to HIV peptides. Such tolerance is exploited to counteract the non-protective or even harmful antibody responses to certain gp120 peptides (Homsy, J. *et al.* (1989) *Science* 244:1357; Finkel, T. *et al.* (1994) *Curr. Opin. Immunol. 6*:605; Füst, G. *et al.* (1995) *Immunol. Today, 16*:167), as described below.

In the C1 region of gp120, both T-cell and B-cell epitopes have been shown to be immunodominant (Abacioglu, Y. *et al.* (1994) *AIDS Res. Human Retrovirus. 10*:371). Within C1, these investigators defined boundaries, termed C1a and C1b. The C1a peptide epitope FNMWKND (corresponding to residues 63-69 of Figure 1, SEQ ID NO:83) can be detected with monoclonal antibody B10. A preferred peptide has the structural motif similar to that used earlier to form a 14-mer with a C-terminal cysteine for coupling ease using MBS (*m*-maleimodobenzoyl-N-hydroxysuccinimide ester) AAAFNMWKNDGGGC (SEQ ID NO:113). This peptide can be chemically conjugated to HGG for evaluation *in vivo* and *in vitro* for tolerogenicity.

Table IV, above provides amino acids sequences of T helper cell epitopes of gp120 that have been identified using either human or murine test systems and have been entered in the HIVMID published on the Los Alamos National Laboratory World Wide Web Site. Preferred fIg constructs include one or more of the epitopes presented in Table IV linked to the N-terminus of an Ig H chain as was described above for B cell epitopes (*e.g.*, those in Table III). However, the present inventors do not intend to be limited by this listing of sequences which are specifically based on the amino acids sequences of HIV subtype B viruses. The art permits identification of other epitopic sequences derived from other HIV subtypes (discussed above) as well as viral isolates or "quasi species" thereof.

TABLE II

Defined Regions Of gp120 For Use As Tolerogenic Epitopes

Region of gp120	Residues (approximate)[1]
C1	1-95
C1 (subregion)	60-90
V1 loop	101-127
V2 loop	144-166
C2	168-269-
C2	223-238
V3 loop	266-301
V3 region	274-288
C3	298-360
C3(subregion)	331-348
V4 loop	355-388
C4	383-427
V5	428-442
C5	443-481 (C-terminus)

[1] See Figure 1 for consensus amino acid sequence of HIV-1 subtype B gp120 (and selected individual amino acid substitution variants in individual viral isolates). See Figure 2 for consensus amino acid sequence of other HIV-1 subtypes

TABLE III

HIV gp120 B Cell Epitopes Identified by mAbs

Region or Positions NO:	Virus Strain*	Amino Acid Residues	SEQ ID
30-51	LAI	ATEKLWVTVYYGVPVWKEATTT	114
31-50	LAI	TEKLWVTVYYGVPVWKEATT	115
31-50	LAI	GVPVWKEATT	116
42-61	LAI	VPVWKEATTTLFCASDAKAY	2
64-78	IIIB	EVHNVWATHACVPTD	3
51-70	LAI	YDTEVHNVWA	4
81-90	LAI	PQEVVLVNVT	5
81-100	LAI	PQEVVLVNVTENFDMWKNDM	6
89-103	IIIB	PNPQEVVLVNVTENF	117
91-100	LAI	ENFDMWKNDM	118
93-96/94-97	LAI/BH10	FNMW	119
94-99	BH10	FNMWKN	120
91-100	LAI	ENFDMWKNDM	118
101-110	LAI	VEQMHEDIIS	121
101-120	LAI	VEQMHEDIISLWDQSLKPCV	122
311-321	HXB10	EQMHEDIISLWDQSLKPCVK	123
101-120	LAI	LWDQSLKPCV	124
102-121	LAI	EQMHEDIISLWDQSLKPCVK	123
114-123	IIIB	MHEDIISLWD	125
122-141	LAI	LTPLCVSLKCTDLKNDTNTN	126
162-169	HXB2	STSIRGKV	127
162-171 V2	BH10	STSIRGKVQ	128
170-180	BH10 or IIIB	QKEYAFFYKLD	129
172-191	HXB2	EYAFFYKLDIIPIDNDTTSY	130
162-181	BH10	STSIRGKVQKEYAFFYKLDI	131
172-181	HXB2	EYAFFYKLDI	132
221-220	LAI	EPIPIHYCAPA	133
211-230	LAI	EPIPIHYCAPAGFAILKCNN	134
222-231	LAI	GFAILKCNNK	135
242-261	LAI	RPVVSTQLLL	136
252-271	LAI	RPVVSTQLLLNGSLAEEEVV	137
257-262	BH10	TQLLLN	138
257-263	BH10	TQLLLNG	139
262-281	LAI	NGSLAEEEVVIRSVNFTDNA	140

Table III, cont.

Region or Positions NO:	Virus Strain*	Amino Acid Residues	SEQ ID
261-280	LAI	VIRSVNFTDN	141
299-304	IIIB	INCTRP	142
299-304	IIIB	SVEINCTRPNNNTRKSI	143
299-308	IIIB	PNNNTRKSIR	7
300-315	HXB10	NNNTRKRIRIQRGPGR	8
304-308	IIIB	RKSIR	9
309-318/329-338	IIIB	IQRGPGRAFV / AHCNISRAKW	144
314-323/494-503	infec	GRAFVTIGKI / LGVAPTKAKR	145
316-322	infec	PGRAFY	12
302-321	BH10	NTRKSIRIQRGPGRAFVTIG	13
306-338	BH10	PNNNTRKSIRIQRGPGRAFVTIGKIGNMRQAHC	14
307-318	IIIB	NNTRKSIRIQRG	15
308-313	MN	NKRKRIHIGPGRAFYTTKNIIGTIC	16
308-313	MN	V3 tip	
304-318	LAI	RKSIRIQRGPGRAFV	17
299-304	IIIB	IRIQRGPGR	18
299-304	IIIB	KRIRIQRGPGRAFVTIG	19
308-328	BRU	QRGPGRAF	20
V3	BRU	RGPGRAFV	21
V3	MN	RKRIHIGPGRAFYTT	22
V3	infec	-I----G--FY-T	146
311-321	HXB10	RGPGRAFVTIG	23
V3	infec	SISGPGRAFYTG	24
V3	MN	KRIHI	25
V3	infec	IXIGPGR	147
V3	MN	KRIHIGP	26
V3	MN (or infec)	IHIGPGR	27
V3	MN	HIGPGR	28
V3	infec	HIGPGRA	29
V3	infec	RKRIHIGPGRAFYTT	22
V3	?	HIGP	30
311-324	MN	RIHIGPGRAFYTTG	31
312-318	MN	IXIGPGR	147
307-316	IIIB	RIQRGPGRAF	32
307-316	IIIB	IQRGPGRAFV	10

Table III, cont.

Region or Positions NO:	Virus Strain*	Amino Acid Residues	SEQ ID
308-316	IIIB	IQRGPGRAF	33
V3	IIIB	IRIQRGPGRAFVTI	34
V3 316-330	HXB2	RGPGRAFVTIGKIG	35
V3	?	QRGPGRA	36
V3	IIIB	IXXGPGRA	37
V3	infec	IGPGR	38
V3	MN	GPGR	39
V3	MN	GPXR	40
308-313	MN	GPGRAF	41
V3	MN	RIHIG	42
V3	MN	HIGPGRAF	43
V3	IIIB	GRAF	44
V3	IIIB	RAF	148
361-380	LAI	IFKQSSGGDPEIVTHSFNCGG	149
362-381	LAI	FKQSSGGDPEIVTHSFNCGGE	150
380-393	LAI	GEFFYCNSTQLFNS	151
C3	HIV2ROD	HYQ [core]	152
C3	HIV2ROD	RNISFKA	153
C3	HIV2ROD	APGK[core]	154
395-400	BH10	WFNSTW	155
423-437	IIIB	IINMWQKVGKAMYAP	156
429-443		EVGKAMYAPPISGQI	157
429-438	BRU	EVGKAMYAPP	158
429-438	BRU	GKAMYAPPIS	159
CD4 bs*	IIIB	AMYAPPI	160
CD4 bs	IIIB	AMYAPPISGQ	161
425-441	IIIB	NMWQEVGKAMYAPPISG	162
412-453	MN	GKAMYAPPIS	159
451-470	LAI	SNNESEIFRL	163
471-490	LAI	GGGDMRDNWRSELYKYKVVK	45
490-508	IIIB	KYKVVKIEPLGVAPTKAKRR	46
314-323 and 494-503 and 47		GRAFVTIGKI and LGVAPTKAKR	11
472-491	LAI	GGDMRDNWRSELYKYKVVKI	48
491-500	LAI	IEPLGVAPTK	49
503-509	infec	RRVVQRE	50
C terminus	infec	PTKAKRR	51
C terminus	infec	VVQREKR	52

TABLE IV
HIV gp120 T Helper Cell Epitopes

Position No.	Virus Strain	Amino Acids	Ref.	SEQ ID NO.
39-51		EQLWVTVYYGVPV	1	53
45-55		VYYGVPVWKEA	1	54
48-61		GVPVWKEATTLFC	1	55
72-82		AHKVWATHACV	1	56
74-85	LAI	NVWATHACVPTD	2	57
81-92		CVPTNPVPQEVV	1	58
108-119	LAI	VEQMHEDIISLW	2	59
109-121		EQMHEDIISLWDQ	1	60
109-123	IIIB	EQMHEDIISLWDQSL	3	61
112-124	IIIB, BH10	HEDIISLWDQSLK	3-9	62
115-126	LAI	IISLWDQSLKPC	2	164
204-216		SVITQACSKVSFE	1	165
215-228		FEPIPIHYCAFPGF	1	166
233-244	LAI	AGFAILKCNNKT	2	167
269-283	IIIB B10	EVVIRSANFTDNAKT	10	168
274-288	IIIB B10	SANFTDNAKTIIVQL	10	169
296-312	LAI	IIVQLNQSVE	2	170
292-300	SF2	NESVAINCT	11	171
	MN	ESVQIN	12	172
303-321	IIIB	CTRPNNNTRKSIRIQRGPG(Y)	13	66
307-322	IIIB	NTRKSIRIQRGPGR	14	67
309-323	IIIB B10	EQRGPGRAFVTIGKI	10	68
315-329	IIIB	RIQRGPGRAFVTIGK	6-8,15,16	69
	MN analog	RIHIGPGRAFYTTKN	16	70
314-328	IIIB B10	GRAFVTIGKIGNMRQ	10	71
324-338	IIIB	FVTIGKIGNMRQAHC	3	173
327-341	HXB2	RQAHCNISRAKWNNT	17	174
342-356	IIIB	RAKWNNTLKQICSKL	3	175
346-359		QIVKKLREQFGNNK	18	176
364-378	IIIB B10	SSGGKPEIVTHSFNC	10	177
368-377	LAI	NKTIIFKQSS	2	178
369-383	IIIB B10	PEIVTHSFNCGGEFF	10	179
394-408	IIIB B10	TWFNSTWSTKGSNNT	10	180
399-413	IIIB B10	TWSTKGSNNTEGSDT	10	181
410-429	PV22	GSDTITLPCRIKQFINMWQE	19,20	182
424-438	IIIB B10	INMWQEVGKAMYAPP	10	183
428-443	IIIB B10	KQIINMWQEVGKAMYA	3-8,12,13,16,21,22	184
432-446	IIIB	NMWQEVGKAMYAPPI	3	185

Table IV, cont.

Positions	Virus Strain	Amino Acids	Ref.	SEQ ID NO.
437-451	IIIB	VGKAMYAPPISGQIR	3	73
459-473	IIIB B10	GNSNNESEIFRPGGG	10	74
466-481		FRPGGGDMRDNWRSEL	18	75
474-488	IIIB B10	DMRDNWRSELYKYKV	10	76
483-497	IIIB	RDNWRSELYKYKVVK	3	77
C492-506	IIIB	CKYKVVKIEPLGVAPT	3	78
484-498	IIIB B10	YKYKVVKIEPLGVAP	10	79
494-518	IIIB	KVVKIEPLGVAPTKAKRRVVQREKRC	14	80

References: 1. K.J. Sastry *et al., AIDS*, 1991 5:699-707; 2. R.D. Schrier *et al., J. Immunol.* 1989 142:1166-1176; 3. P.M. Hale *et al.*, Int'l. Immunology, 1989 1:4:409-415; 4. K.B. Cease *et al., Proc. Natl. Acad. Sci. USA*, 1987 84:4249-4253; 5. J.A. Berzofsky *et al., Nature*, 1988 334:706-708; 6. M. Clerici *et al., Nature*, 1989 339:383-385; 7. M. Clerici *et al., J. Immunol.*, 1991 146:2214-2219; 8. M. Clerici *et al., Eur. J. Immunol.*, 1991 21:1345-1349; 9. A. Hosmalin *et al., J. Immunol.*, 1991 146:1667-1673; 10. B. Wahren *et al., Vaccines*, 1989 89:89-93; 11. P. Botarelli *et al., J. Immunol.*, 1991 147:3128-3132; 12. F.D.M. Veronese *et al., J. Mol. Biol.*, 1994 243:167-172; 13. T. J. Palker *et al., J. Immunol.*, 1989 142:3612-3619; 14. G. Goodman-Snitkoff *et al., Vaccine*, 1990 8:257-262; 15. H. Takahashi *et al., J. Exp. Med.*, 1990 171:571-576; 16. M. Clerici *et al., J. Inf. Dis.*, 1992 165:1012-1019; 17. A. P. Warren *et al., AIDS Res. Hum. Retrovir.*, 1995 8:559-564; 18. J. Krowka *et al., J. Immunol.*, 1990 144:2535-2540; 19. K. M. Callahan *et al., J. Immunol.*, 1990 144:3341-3346; 20. M. Polydefkis *et al., J. Exp. Med.*, 1990 171:875-887; 21. B.F. Haynes *et al., J. Exp. Med.*, 1993 177:717-727; 22. D. M. Klinman *et al., AIDS Res. Hum. Retrovir.*, 1995 11:97-105

Preferred approaches to identification and selection of T cell epitopes, in particular T helper cell epitopes, for inclusion in a flg as described herein, utilize computer-based algorithms. Several computer-driven algorithms have been devised in the art which exploit the alphabetic representation of amino acid sequence information to search for T cell epitopes by searching the amino acid sequence of a given protein for characteristics believed to be common to immunogenic peptides, and thereby locating regions that are likely to induce cellular immune response *in vitro*. With the rapid expansion of sequence data on geographic subtypes (clades) of HIV and individual HIV quasi-species, the application of these algorithms to HIV proteins can significantly reduce the number of regions which would require *in vitro* testing for the desired property (generally immunogenicity) although as envisioned by the present inventors, the desired property is tolerogenicity when presented to the immune system as an flg. Computer-driven algorithms can identify regions of HIV proteins

that contain epitopes and are less variable among geographic HIV isolates; alternatively, computer-driven algorithms can rapidly identify regions of each geographic isolate's more variable proteins that should be included in a multi-clade tolerogenic flg.

Well-known and conventional ways to identify T cell epitopes within protein antigens, which may be used for the present invention, employ a variety of methods, including the use of whole and fragmented native or recombinant antigenic protein, and the "overlapping peptide" method. This approach involves synthesis of overlapping peptides which span the entire sequence of a given protein antigen, in the present case, gp120. These overlapping peptides are then tested for their capacity to stimulate the relevant T cell responses *in vitro*, for example T cell proliferative responses (Vordermeier, H.M. *et al.* (1993) *Immunology 80*:6-12; Ashbridge, K.R. *et al.* (1992) *J. Immunol.* 148:2248-2255). While the overlapping peptide method is thorough, it is both cost- and labor-intensive.

The computer based algorithm methods minimize the cost and labor of the overlapping peptide method and avoid the potential omission of sites between overlapping fragments. Such computer-based algorithms designed to predict T cell epitopes from the amino acid sequences of proteins include AMPHI. AMPHI searches a protein's primary structure for peptides with a high probability of folding as amphipathic structures (Margalit, H. *et al.* (1987) *J. Immunol.* 138:2213-2229; Cornette, J.L. *et al.* In: *The Amphipathic Helix* (Ed. Epand, R.M.), CRC Press, Boca Raton, 1993). Seventy percent of published epitopes were found to contain sequences that would have been predicted by AMPHI (Margalit *et al.*, *supra*; Spouge, J.L. *et al.* (1987) *J. Immunol.* 138:204-212). The number of known T cell epitopes has quadrupled since the design of AMPHI, and of these, 65% are amphipathic, such that the correlation remains highly significant (Cornette *et al.*, *supra*). Other epitope prediction algorithms which analyze protein sequences for specific secondary structural or sequence characteristics (Stille, C.J. *et al.* (1987) *Mol. Immunol. 24*:1021-1027; Rothbard, J.B. *et al.* (1988) *EMBO J. 7*:93-100; Salomon, M. *et al.*,

(1993) Vaccine 11:1067-1073) generally search for a spacing of hydrophobic residues similar to that searched for by the AMPHI algorithm.

DeGroot and colleagues (Meister, G.E. *et al.* (1995) *Vaccine*, 13581-591) developed two computer-based algorithms for T cell epitope prediction, OptiMer and EpiMer, which incorporate current knowledge of MHC-binding motifs. OptiMer locates amphipathic segments of protein antigens with a high density of MHC-binding motifs. EpiMer identifies peptides with a high density of MHC-binding motifs alone. These algorithms exploit the tendency for MHC-binding motifs to cluster within short segments of each protein. Epitopes predicted by these algorithms contain motifs corresponding to many different MHC alleles, and may contain both class I and class II motifs, features thought to be ideal for the peptide components of synthetic subunit vaccines. Use of these two algorithms provide sensitive and efficient means for the prediction of promiscuous T cell epitopes that may be used to development preparations such as epitope-specific vaccines, or, for the present application, specific tolerogenic epitopes to be used in an flg.

OptiMer examines known amino acid sequences of proteins and generates a list of peptides that contain these motifs; the algorithm then identifies peptides that would be amphipathic if folded as a helix or twisted as a beta-strand, using the AMPHI algorithm. These potentially amphipathic peptides are compared to the list of MHC-binding motif matches. OptiMer extends the predicted amphipathic peptides, to maximize the density of MHC-binding motif matches per length of protein region.

The EpiMer algorithm searches protein amino acids sequences for MHC-binding motif matches, generating a list of matches for each protein. The relative density of these motif matches is determined along the length of the antigen, resulting in the generation of a motif-density histogram. Finally, the algorithm identifies protein regions in this histogram with a motif match density above an algorithm-defined cutoff density value, and produces a list of subsequences representing these clustered, or motif-rich regions. The regions selected by EpiMer may be more likely to act as multi-determinant binding peptides than randomly chosen peptides from the same antigen, due to their concentration of MHC-binding motif matches.

OptiMer and EpiMer, have been used to predict putative epitopes in five *Mycobacterium tuberculosis* (Mtb) protein antigens (14 kDa, 16 kDa, 19 kDa, 38 kDa, and 65 kDa) and three human immunodeficiency virus (HIV) protein antigens (nef, gp160 which is the precursor of gp120 and gp41, and reverse transcriptase (RT). To evaluate the new algorithms' predictive power, Meister *et al.* compared OptiMer- and EpiMer-predicted epitopes, AMPHI-predicted epitopes, and peptides that would have been synthesized using the "overlapping peptide" method, to a selection of published T cell epitopes for the above proteins. These algorithms were used to predict T cell epitopes from within the published sequences of three HIV protein antigens. Epitopes published for the HIV protein antigens nef and gp160 were almost exclusively class I MHC-restricted, while epitopes published for RT were both class I- and class II-restricted.

A version of either OptiMer or EpiMer based on the list of class I-restricted MHC-binding motifs was used to predict putative epitopes for nef and gp160, while versions of both algorithms based on the combined list of class I- and class II-restricted motifs were employed to predict putative epitopes for the HIV protein antigen RT. In all, 29 putative epitopes were generated by the class I-specific version of OptiMer (totaling 661 amino acids in length); 30 putative epitopes were generated by EpiMer, totaling 614 amino acids in length. AMPHI generated 36 putative epitopes (totaling 666 amino acid residues), and 104 peptides (totaling over two thousand residues in length) would have been required by the overlapping peptide method. For these two HIV protein antigens, the class I-restricted implementations of both OptiMer and EpiMer identified published epitopes with an efficiency comparable to that of AMPHI, and greater than that of the overlapping peptide method. EpiMer's sensitivity per amino acid exceeds that of either OptiMer or AMPHI. For RT, the combined class I/class II implementation of OptiMer generated 18 putative epitopes (totaling 422 amino acids); the same implementation of EpiMer generated 22 putative epitopes (totaling 361 amino acids in length). These values compare with 23 putative epitopes generated by AMPHI (totaling 433 amino acids) and 55 peptides (totaling over one thousand residues) required by the overlapping peptide method. OptiMer

and EpiMer predict published T cell epitopes for the HIV protein RT with both efficiency and sensitivity comparable to that of the AMPHI algorithm. EpiMer again attains the highest sensitivity per amino acid of these three algorithms. In a recent comparison of EpiMer predictions to published HIV protein T cell epitopes, the EpiMer algorithm was shown to be 2.4-fold more sensitive (per amino acid residue) than the overlapping peptide method for detecting published T cell epitopes for four HIV proteins, gp160, nef, tat, and gag. In contrast, AMPHI was somewhat less sensitive (1.6-fold) (Roberts, C.G.P. *et al.* (1996) *AIDS Res. Human Retrovir.* 12:593-607). A summary of comparisons of the overlapping peptide method with the AMPHI and EpiMer prediction method is provided by Roberts *et al.* (*supra*).

The above approach to HIV epitopes has been embodied in an algorithm recently named EpiMatrix/HIV which predicts the sequences most likely to bind to MHC molecules when given a number of primary HIV protein sequences and which was developed by A.S. De Groot at Brown University and implemented for the Internet by AVX Design Inc., Providence, Rhode Island. Both a website and an online tool, EpiMatrix is located on the Internet at http://www.epimatrix.com/hiv as of November 1, 1996. Use of this algorithm in accordance with the present invention allows selection of peptides that are highly likely to bind to a particular subject's MHC, thereby enabling identification of T-helper epitopes (as well as cytotoxic T-cell epitopes for vaccine development). The EpiMatrix algorithm yields a score for each peptide in a 10-mer frame. Scoring is a quantitative estimate of the likelihood (relative to other sequences) that a peptide will bind to a given HLA molecule. Two scoring methods are used: single-allele predictions score for specific HLA alleles and clustered predictions score peptides by the prevalence of MHC alleles in selected populations Matrices for all of the major (greater than 10% population prevalence) MHC alleles representing world populations are included in the algorithm (B.M. Jesdale *et al., Vaccines '97*, Cold Spring Harbor Laboratory Press). EpiMatrix reduces the total number of regions of HIV proteins to be evaluated *in vitro*, permitting more rapid identification of desired epitopes. (See, also *AIDSWEEKLY Plus*, 18 November 1996 issue).

Additional MHC binding motif-based algorithms have been described by K.C. Parker *et al.* (*J. Immunol.* (1994) *152*:163-175) and Y. Altuvia *et al.* (1995) *J. Mol. Biol. 249*:244-250). In these algorithms, binding to a given MHC molecule is predicted by a linear function of the residues at each position, based on empirically defined parameters, and in the case of Altuvia *et al.*, known crystallographic structures are also taken into consideration. J. Hammer *et al.* (*J. Exp. Med.* (1994) *180*:2353-2358) described a technique known as "peptide side chain scanning" which is used to predict binding peptides for an MHC allele.

The EpiMer/EpiMatrix algorithm predicted putative T cell epitopes from protein sequences for HIV-1 nef, gp160, gag p55, and tat that required fewer peptides and therefore fewer amino acid residues to be synthesized than either AMPHI-predicted peptides or overlapping peptides. For the four HIV-1 proteins, EpiMer predicted 43 peptide epitopes, AMPHI predicted 68 peptides , and the overlapping peptide method (20 amino acid long peptides overlapping by 10 amino acids) would have required 161 peptides. Details (amino acid start and stop, number of MHC binding motifs) of the predicted proteins are available36. Regions of HIV proteins that contain as many as 20 to 30 MHC binding motifs can be identified using this algorithm.

The various known methods for epitope prediction are not mutually exclusive. As the contributions of side chains and tertiary peptide structure to peptide-MHC binding are better quantified, the development of a computer algorithm that predicts T cell epitopes based on a matrix of side chain information such as one described by J. Hammer (1995) *Curr. Opin. Immunol. 7*:263-269) will become available. The identification of novel structural features which are able to independently predict peptide recognition and their subsequent synthesis into a combined algorithm with statistically verifiable predictive capacity, allows a dramatic reduction in the time and effort required to synthesize and test potential T cell antigenic sites for HIV proteins, by allowing the prediction of sites with a high concentration of antigenic features.

HIV protein regions that contain multiple overlapping class-II restricted epitopes, also known as "multi-determinant" or multi-determinant peptides, have been identified in mice and humans. Such regions might be important to include in the

synthesis of an fIg having multiple tolerogenic T helper cell epitopes as described herein. This is particularly useful if a multi-determinant T cell epitope is involved in stimulating antibody responses (*i.e.*, to B cell epitopes).

Table V, below presents a list of epitopes of gp120 (and several N-terminal epitopes of gp41) which were identified using EpiMer (Roberts *et al., supra*). These sequences are from the BH10 strain of HIV-1. The amino acid sequence of this HIV strain was obtained from the SWISS-PROT protein sequence data bank , Accession No. P03375 (EMBL Data Library, Heidelberg, Germany). The residue numbers shown in Table V are from this sequence bank. Those residues beyond amino acid 511 are part of gp41, not gp120. In a preferred embodiment, the present invention provides a tolerogenic fIg H chain or intact fIg molecule which includes at the N-terminus of the H chain one or more of the HIV peptide epitopes listed in Table V.

TABLE V

T Cell Epitopes of Hiv gp120 Identified by Epimer Algorithm

AMINO ACIDS	SEQUENCE
19 - 34	**TMLLGMLMICSATEKL** (SEQ ID NO:186)
20 - 28	MLLGMLMIC
21 - 29	LLGMLMICS
21 - 30	LLGMLMICSA
22 - 29	LGMLMICS
22 - 30	LGMLMICSA
24 - 32	MLMICSATE
24 - 33	MLMICSATEK
25 - 33	LMICSATEK
26 - 34	MICSATEKL
36 - 54	**VTVYYGVPVWKEATTTLFC** (SEQ ID NO:63)
36 - 44	VTVYYGVPV
36 - 45	VTVYYGVPVW
37 - 46	TVYYGVPVWK
38 - 46	VYYGVPVWK
39 - 46	YYGVPVWK
42 - 51	VPVWKEATTT
44 - 52	VWKEATTTL
44 - 53	VWKEATTTLF
45 - 53	WKEATTTLF
45 - 54	WKEATTTLFC
84 - 95	**VVLVNVTENFNM** (SEQ ID NO:64)
85 - 93	VLVNVTENF
85 - 94	VLVNVTENFN
87 - 95	VNVTENFNM
115 - 127	**SLKPCVKLTPLCY** (SEQ ID NO:65)
116 - 124	LKPCVKLTP
116 - 125	LKPCVKLTPL
117 - 125	KPCVKLTPL
119 - 127	CVKLTPLCV

AMINO ACIDS	SEQUENCE
168 - 185	**KVQKEYAFFYKLDIIPID** (SEQ ID NO:187)
168 - 176	KVQKEYAFF
169 - 177	VQKEYAFFY
169 - 178	VQKEYAFFYK
171 - 179	KEYAFFYKL
173 - 181	YAFFYKLDI
173 - 182	YAFFYKLDII
174 - 181	AFFYKLDI
174 - 182	AFFYKLDII
175 - 182	FFYKLDII
175 - 183	FFYKLDIIP
175 - 184	FFYKLDIIPI
176 - 184	FYKLDIIPI
198 - 214	**TSVITQACPKVSFEPIP** (SEQ ID NO:188)
198 - 207	TSVITQACPK
199 - 207	SVITQACPK
199 - 208	SVITQACPKV
200 - 208	VITQACPKV
202 - 210	TQACPKVSF
204 - 213	ACPKVSFEPI
205 - 213	CPKVSFEPI
249 - 261	**HGIRPVVSTQLLL** (SEQ ID NO:189)
249 - 256	HGIRPVVS
251 - 259	IRPVVSTQL
251 - 260	IRPVVSTQLL
252 - 260	RPVVSTQLL
284 - 296	**IIVQLNQSVEINC** (SEQ ID NO:190)
284 - 292	IIVQLNQSV
285 - 293	IVQLNQSVE
286 - 294	VQLNQSVEI
286 - 295	VQLNQSVEIN
288 - 296	LNQSVEINC

TABLE V, cont.

AMINO ACIDS	SEQUENCE
310 - 330	**QRGPGRAFVTIGKIGN MRQAH** (SEQ ID NO:191)
312 - 320	GPGRAFVTI
314 - 322	GRAFVTIGK
315 - 322	RAFVTIGK
315 - 323	RAFVTIGKI
317 - 325	FVTIGKIGN
318 - 326	VTIGKIGNM
318 - 327	VTIGKIGNMR
319 - 327	TIGKIGNMR
320 - 328	IGKIGNMRQ
320 - 329	IGKIGNMRQA
351 - 363	EQFGNNKTIIFKQ (SEQ ID NO:192)
351 - 359	EQFGNNKTI
353 - 361	FGNNKTIIF
353 - 362	FGNNKTIIFK
381 - 392	**EFFYCNSTQLFN** (SEQ ID NO:193)
382 - 390	FFYCNSTQL
382 - 391	FFYCNSTQLF
383 - 391	FYCNSTQLF
383 - 392	FYCNSTQLFN
414 - 445	**ITLPCRIKQIINMWQEV GKAMYAPPISGQIRC** (SEQ ID NO:81)
414 - 422	ITLPCRIKQ
414 - 423	ITLPCRIKQI
416 - 424	LPCRIKQII
416 - 426	LPCRIKQIINM
418 - 426	CRIKQIINM
420 - 428	IKQIINMWQ
420 - 428	IKQIINMWQ
420 - 429	IKQIINMWQE
424 - 433	INMWQEVGKA
426 - 435	MWQEVGKAMY
427 - 435	WQEVGKAMY
428 - 436	QEVGKAMYA

AMINO ACIDS	SEQUENCE
482 - 527	**ELYKYKVVKIEPLGVA PTKAKRRVVQREKRAV GIGALFLGFLGAAG** (SEQ ID NO:194)
482 - 490	ELYKYKVVK
483 - 491	LYKYKVVKI
483 - 492	LYKYKVVKIE
486 - 494	YKVVKIEPL
486 - 495	YKVVKIEPLG
488 - 496	VVKIEPLGV
488 - 497	VVKIEPLGVA
489 - 497	VKIEPLGVA
491 - 499	IEPLGVAPT
493 - 502	PLGVAPTKAK
494 - 502	LGVAPTKAK
495 - 503	GVAPTKAKR
495 - 504	GVAPTKAKRR
496 - 504	VAPTKAKRR
496 - 505	VAPTKAKRRV
497 - 505	APTKAKRRV
500 - 508	KAKRRVVQR
503 - 511	RRVVQREKR
505 - 513	VVQREKR/AV*
506 - 514	VQREKR/AVG
506 - 515	VQREKR/AVGI
507 - 515	QREKR/AVGI
510 - 518	KR/AVGIGAL
511 - 519	R/AVGIGALF

* "/" follows the C terminal residue of gp120

Application of the above approach to general HIV tolerogenic preparations may be restricted by the amount of sequence variation in individual quasi-species, HIV strains, and HIV subtypes as well as by the MHC background of the subject population. For example, the region of amino acids at about 130 to 160 (depending upon which subtype or isolate), has a great deal of inter-strain variation and may therefore best be avoided in designing a tolerogenic fIg which has the broadest range of applicability across virus variants and subtypes. HIV peptide epitopes which contain multiple MHC binding motifs, either conserved across HIV strains or derived from several different HIV strains, may be ideal candidates for targeting for T helper cell-directed tolerance induction, as it is assumed that the tolerogen will be presented *in vivo* by host MHC molecules. Thus, epitopes with multiple MHC binding motifs or having an MHC binding motif present in the highest frequency in the subject population (race, ethnic group, e.) would be preferably selected for inclusion in a tolerogenic fIg. The EpiMer algorithm is particularly well suited for identifying and selecting such epitopes.

Preparation of Recombinant fIg and its Transfer

The present invention provides polynucleotides encoding the fIg in the form of recombinant DNA molecules in vehicles such as plasmid and retroviral vectors, capable of expression in a desired eukaryotic host cell as disclosed herein. The invention also provides hosts transfected or transduced with the fIg constructs which are capable of producing in culture or *in vivo* the fIg molecules and secreting them or displaying them on the cell surface.

A preferred engineering strategy for inserting a foreign epitope at the N-terminus of an IgG γ chain is shown in Figure 4A and 4B. Figure 4A depicts the incorporation of an oligonucleotide, in this example encoding the λ phage C1 repressor peptide 12-26. However, the present invention exploits the same general scheme wherein a native or synthetic gp120 peptide epitope is inserted in place of the 12-26 peptide. This is illustrated in Figure 4B. Any Ig gene construct may be used for insertion of the tolerogenic epitope or epitopes. A preferred Ig gene encodes human Ig, more preferably an Ig comprising a human γ chain.

The general principles of recombinant DNA technology are utilized, as described for example, in Sambrook, J. *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989; Ausubel, F.M. *et al. Current Protocols in Molecular Biology*, Vol. 2, Wiley-Interscience, New York, 1987; Lewin, B.M., *Genes IV*, Oxford University Press, Oxford, (1990); Watson, J.D. *et al.*, *Recombinant DNA*, Second Edition, Scientific American Books, New York, 1992, which references are hereby incorporated by reference in their entirety.

The DNA construct encodes an individual fIg H chain, although the protein products of this invention include both the fIg H chain and a complete assembled Ig molecules comprising the fIg H chain having one or more HIV gp120 epitopes in combination with a native human Ig L chain. The fIg may also comprise two different H chains, one of which is a fusion protein having one or more HIV gp120 epitopes added to or included in the V region.

Genetic sequences, especially cDNA sequences, encoding either a complete fIg H chains, the fIg V regions or a human Ig C region of any Ig isotype, most preferably, an IgG isotype (*i.e.*, a human Cγ chain) are also provided herein.

The invention also provides a genetic sequence, especially a cDNA sequence encoding an Ig V region fusion protein in which the V region encoding DNA has been combined in frame with one or more HIV gp120 epitopes. Though, genomic DNA sequences may also be used, cDNA sequences are particularly preferred.

One non-limiting approach to producing the fIg comprises the steps of:

1. Selection of one or more gp120 epitopes as described below for which tolerance is desired;
2. Preparation of DNA encoding the epitope or epitopes selected above; this can be done by isolating HIV RNA and cloning an preparing cDNA corresponding to all or part of gp120, by isolating and cloning DNA from HIV-infected cell, or if the DNA is sufficiently short, synthesizing an oligonucleotide having the desired coding sequence. The latter synthetic approach permits construction of artificial

combinations of two or more gp120 epitopes or which are not contiguous in the native protein.

3. Introduction of appropriate restriction enzyme recognition sites in the gp120 DNA to permit ligation to Ig H chain encoding DNA, preferably human γ chain-encoding DNA; this can be done by PCR, site-directed mutagenesis or synthetically;
4. Selection of endogenous restriction sites of the Ig-encoding DNA or modification of the DNA as above to introduce restriction sites corresponding to those in the gp120 DNA such that they can form cohesive ends and be ligated
5. Ligation of the gp120 DNA to the Ig H chain DNA using conventional methods.
6. Expression and production of the fIg H chains or intact Ig molecules (H_2L_2) in a selected host, preferably human lymphoid or hematopoietic cells.

Oligonucleotides which can be used as primers for introducing useful restriction sites into the gp120 and human Ig DNA for subsequent linkage are well known in the art. See, for example, Sambrook *et al., supra.*

In an alternate embodiment, rather than using DNA encoding an entire Ig H chain, the gp120 DNA is linked to an Ig V gene cassette. Because the antibody specificity of the fIg is not important, any V region DNA can be selected. A preferred V gene is one which encodes a protein which, after fusion of a gp120 epitope or epitopes, even a full length gp120 protein, still maintains its ability to fold properly in an full Ig molecule (H_2L_2).

The variable (V) domain of an Ig chain includes hypervariable (HV) regions which are also known as complementarity-determining regions (CDRs) because they are important in "determining" the structure of the antibody combining site that is complementary the epitope bound. Each H and L chain V region has three HVs or CDRs. The segments on either side of each HV region which are relatively invariant are termed "framework regions" (FRs). Thus, the order of these regions in a V domain (from the N-terminus)is as follows: FR1-HV1-FR2-HV2-FR3-HV3-FR4. For example, the three HV regions are roughly from residues 28-35, 49-59 and 92-103, respectively.

The framework regions form the β sheets that provide th structural framework of the domain, with the HV sequences corresponding to three loops at one edge o each sheet that are juxtaposed in the folded protein. The HV loops from the V_H and V_L domains are brought together, creating a single HV site at the tip of the Fab fragment which forms the antigen binding site. (See, for example, Janeway, C.A., Jr. *et al.*, *IMMUNOBIOLOGY,* 2n ed., Garland Publishing Inc., New York, 1996, chapter 3).

The first framework region (FR1) is the most N-terminal of the V region. Eisen, H.N., *GENERAL IMMUNOLOGY,* (J. Lippincott Co., Philadelphia, 1990) at pages 57-59, in particular Figure 14-19 at page 58, shows the amino acid sequences of the first framework region of 5 different human H chains. The first framework region includes the 30 N-terminal amino acids at which point the HV1 region follows. A framework region of nine different human κ L chains belonging to three different groups VκI, VκII and VκIII are shown in this textbook figure.. Again, the FRs are about 30 residues, with a number of positions in each group serving as "framework residues" which serve to characterize each Vκ group. In the present invention, the heterologous epitope of the fIg is preferably inserted immediately N terminal to the first framework region. In other embodiments, it may be fused "deeper" into the Ig sequence within the V region.

A spacer comprising between about 1 and 10 amino acids, preferably about 3-5 residues, can be present between the C terminal residue of the heterologous epitope(s), preferably a gp120 epitope(s) and the N terminal residue of the Ig V region, provided that the protein can fold properly to present the gp120 epitope while maintaining its tolerogenic properties. In a preferred arrangement, as exemplified below, a repeat of the 5 N-terminal amino acids of the Ig H chain is inserted N-terminal from the added the gp120 peptide (or peptides) such that this pentapeptide sequence is repeated on either end of the inserted gp120 sequence. If more than one gp120 peptide is included, a spacer as described herein may be linked to one or more of the added gp120 peptides. A major purpose of the spacer is to permit unimpeded folding and proteolytic processing of the fIg as if it were an normal Ig protein. This

assures proper surface expression of the fIg and association with MHC proteins on the surface of a tolerogen-presenting cell.

Advantage can be taken of the natural antigen-binding property of the V region, as is described below for the model murine constructs wherein the V region was specific for the NIP hapten. A complete Ig H chain is constructed by combining the now altered V gene construct containing additional gp120 DNA with a C gene construct encoding a desired human C region, preferably a human Cγ protein. The most preferred C region would be that encoding the γ_3 isotype.

Ig H chain (or V_H) cDNA vectors are typically prepared from human cells and modified by site-directed mutagenesis to place a restriction site at the position in the human sequence in which the gp120 DNA is to be grafted. Preferably this is 5' to the nucleotide encoding the N-terminus of the Ig H chain or the V_H protein.

Two coding DNA sequences are said to be "operably linked" if the linkage results in a continuously translatable sequence without alteration or interruption of the triplet reading frame. A DNA coding sequence is operably linked to a gene expression element if the linkage results in the proper function of that gene expression element to result in expression of the coding sequence.

Expression vehicles include plasmids or other vectors, such as retroviral vectors. A preferred vehicle carries a functionally complete human V_H and C_H having appropriate restriction sites engineered so that any gp120-encoding nucleotide sequence with appropriate cohesive ends can be conveniently ligated thereto. These vehicles can be used as intermediates for propagation of DNA encoding any desired H chain (V_HC_H) ready to receive a gp120 DNA sequence, and for the expression of the complete fIg (gp120-V_HC_H).

Preferred hosts are mammalian cells, most preferably human cells, grown *in vitro* for prolonged periods, or taken from a host, cultured *in vitro* for purposes of transfection and then reintroduced into the host. Mammalian cells provide post-translational modifications to the Ig protein molecules including leader peptide removal, folding and assembly of H and L chains, glycosylation of the protein chains and secretion of the complete functional fIg protein. Mammalian cells which may be

useful as hosts for the production of fIg proteins include cells of lymphoid origin, such as the hybridoma Sp2/O-Ag14 (ATCC CRL 1581) or the myeloma P3X63Ag8 (ATCC TIB 9), also abbreviated as P3, and its derivatives. A preferred murine cell line for expressing the fIg of this invention is J558L. Any cell line which allows for efficient expression and secretion of the fIg constructs of the present invention and which promotes proper folding of the fIg is preferred. Known human lymphoid or hematopoietic cell lines may be used, including B lymphoblastoid lines, lymphomas, hybridomas or heterohybridomas. Examples of cell lines and approaches for expression of recombinant or chimeric or hybrid or modified Ig genes are described in Shin, S.U. *et al.*, (1993) *Int. Rev. Immunol. 10*:177-186; Wright, A. *et al.*, (1992) *Crit. Rev. Immunol. 12*:125-168; Shin, S.U. *et al.* (1992) *Immunol. Rev. 130*:87-107; Morrison, S.L., (1992) *Annu. Rev. Immunol. 10*:239-265; Morrison, S.L. *et al.*, (1989) *Adv. Immunol. 44*:65-92; Weidle *et al.*, (1987) *Gene 51*:21; Whittle *et al.*, (1987) *Protein Engineering 1*:499; Morrison, S.L., (1985) *Science 229*:1202-1207; Morrison S.L. *et al.*, (1984) *Annu. Rev. Immunol. 2*:239-256, all of which references are incorporated by reference in their entirety. In a preferred embodiment, human hematopoietic cells obtained from the intended recipient or those histocompatible with the recipient are transfected with the fIg DNA construct.

Many vector systems are available for the expression of cloned Ig H and L chain genes in mammalian cells (see Glover, D.M., ed.(1985) *DNA Cloning*, Vol. II, pp143-238, IRL Press). Different approaches can be followed to obtain complete H_2L_2 antibodies. It is possible to co-express H and L chains in the same cells to achieve intracellular association and linkage of H and L chains into complete tetrameric H_2L_2 antibodies. The co-expression can occur by using either the same or different plasmids in the same host. Genes for both H and L chains can be placed into the same plasmid, which is then transfected into cells, thereby selecting directly for cells that express both chains. Alternatively, cells may be transfected first with a plasmid encoding one chain, for example the L chain, followed by transfection of the resulting cell line with an H chain plasmid containing a second selectable marker. Cell lines producing H_2L_2 molecules via either route could be transfected with

plasmids encoding additional copies of H, L, or H plus L chains in conjunction with additional selectable markers to generate cell lines with enhanced properties, such as higher production of assembled H_2L_2 antibody molecules or enhanced stability of the transfected cell lines.

One particular strategy for inserting an HIV peptide sequence at or near the N-terminus of an Ig H chain is related to that described in Hebell, T. *et al.* (1991) *Science 254*:102-105 and Ballard, D.W. *et al.* (1993) *Proc. Natl Acad. Sci. USA 83*:9626-9630. A first plasmid is constructed which preferably includes .a full genomic sequence of the Ig H chain and selectable markers, for example, neomycin and or/ampicillin resistance genes. The source DNA encoding the HIV gp120 epitope or epitopes PCR is amplified to create the DNA encoding the desired single or multiple epitopes. Appropriate restriction sites are included on the primers so that the epitope-encoding DNA can be spliced into the Ig gene-containing vector. The gp120 epitope sequence is subcloned into a site, preferably the V_H site of the first plasmid. Recombinant clones are analyzed for proper orientation and polymerase induced errors by double stranded DNA sequencing methods (e.g., Sequenase® kit from U.S. Biochemical).

The promoter sequences useful for the DNA constructs of the of the present invention are any promoters which allow efficient expression of the flg DNA of the invention in a target cell of choice, for example a hematopoietic progenitor cell or a lymphoid cells, more preferably a B cell. Preferred promoters are the promoters of the Ig gene into which the foreign epitope-encoding DNA is being inserted. However, other known promoters of either eukaryotic or viral origin may be used. Suitable promoters are inducible or repressible or, more preferably, constitutive. Examples of useful eukaryotic/viral promoters include the promoter of the mouse metallothionein I gene (Hamer, D., *et al.* (1982) *J. Mol. Appl. Gen. 1*:273-288); the TK promoter of Herpes virus (McKnight, S. (1982) *Cell 31*:355-365); the SV40 early promoter (Benoist, C., *et al.* (1981) *Nature 290*:304-310); and the yeast *gal4* gene promoter (Johnston, S.A., *et al.* (1982) *Proc. Natl. Acad. Sci. (USA) 79*:6971-6975; Silver, P.A.,

et al. (1984) *Proc. Natl. Acad. Sci. (USA) 81*:5951-5955). Strong promoters are most preferred.

The flg construct into which the gp120 epitope(s) has been inserted is introduced ("gene transfer") into the appropriate target cells by conventional methods, *e.g.*, direct physical transfer of plasmid DNA, or preferably, by virus-mediated transfer, for example using a retroviral vector, as discussed below.

A number of means for transferring genes are known in the art and may be used herein, including, for example, electroporation and lipofection. A preferred, and relatively efficient means for achieving transfer of genes is by retrovirus-mediated gene transfer (Gilboa, E. (1987) *Bio-Essays 5*:252-258; Williams, D.A. *et al.* (1984) *Nature 310*:476-480; Weiss, R.A. *et al.*, *RNA Tumor Viruses*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1985). One class of retroviruses, recombinant amphotropic retroviruses have been used as vectors for the transfer of genes into human cells (Cone, R.D. *et al.* (1984) *Proc. Natl. Acad. Sci. USA 81*:6349-6353; Danos, O. *et al.* (1988) *Proc. Natl. Acad. Sci. USA 85*:6460-6464.

When the targets for gene therapy are bone marrow or blood stem cells, for example, it may be advantageous to manipulate the cells *in vitro* with cytokines and then to infect them with the vector bearing the flg gene (Wilson, J.M. *et al.* (1990) *Proc. Natl. Acad. Sci. USA 87*:8437-8441). Recombinant amphotropic retroviruses have been recognized as useful vectors for transferring genes efficiently into human cells, for example to correct enzyme deficiencies (Cone, R.D. *et al.* (1984) *Proc. Natl. Acad. Sci. USA 81*:6349-6353; Danos, O. *et al.* (1988) *Proc. Natl. Acad. Sci. USA 85*:6460-6464). For safety reasons, it is important that a retroviral vector used for gene therapy be capable of infecting only desired cells and not cause generalized infection of cells throughout the body of the individual being treated. In the past, this has generally been accomplished by using helper-defective virus preparations, or mutants lacking the ψ packaging sequence, *etc.*

Another viral vector system useful for this invention is the recombinant adeno associated viral (AAV) transduction system (Lebkowski, J.S., *et al.* (1988) *Mol. Cell. Biol. 8*:3988-3996). AAV DNA integrates into cellular DNA as one to several tandem

copies joined to cellular DNA through inverted terminal repeats (ITRs) of the viral DNA. (Kotin, R.M., *et al.* (1990) *Proc. Natl. Acad. Sci. USA 87*:2211-2215). The transgene DNA size limitation and packaging properties are the same as with any other DNA viral vectors. AAV is a linear single stranded DNA parvovirus, and requires co-infection by a second unrelated virus in order to achieve productive infection. AAV carries two sets of functional genes: *rep* genes, which are necessary for viral replication, and structural capsid protein genes (Hermonat, P.L., *et al.* (1984) *J. Virol. 51*:329-339). The *rep* and *capsid* genes of AAV can be replaced by a desired DNA fragment to generate AAV plasmid DNA. Transcomplementation of rep and capsid genes are required to create a recombinant virus stock. Upon transduction using such virus stock, one recombinant virus uncoats in the nucleus and integrates into the host genome by its molecular ends.

Liposomes may be used to encapsulate and deliver a variety of materials to cells, including nucleic acids and viral particles (Faller, D.V. *et al.* (1984) *J. Virol. 49*:269-272). Preformed liposomes that contain synthetic cationic lipids form stable complexes with polyanionic DNA (Felgner, P.L., *et al.* (1987) *Proc. Natl. Acad. Sci. USA 84*:7413-7417). Cationic liposomes, liposomes comprising some cationic lipid, that contained a membrane fusion-promoting lipid dioctadecyldimethyl-ammonium-bromide (DDAB) efficiently transfer heterologous genes into eukaryotic cells (Rose, J.K., *et al.* (1991) *Biotechniques 10*:520-525). Cationic liposomes can mediate high level cellular expression of transgenes, or mRNA, by delivering them into cultured cell lines (Malone, R., *et al.* (1989) *Proc. Natl. Acad. Sci. USA 86*:60776081).

Ecotropic and amphotropic packaged retroviral vectors infect cultured cells in the presence of cationic liposomes, such as Lipofectin (BRL, Gaithersburg, MD), and in the absence of specific receptors (Innes, C.L. *et al.* (1990) *J. Virol. 64*:957-961).

Physical means well-known in the art can be used for direct gene transfer, including administration of plasmid DNA (Wolff *et al.*, 1990, *supra*) and particle-bombardment mediated gene transfer, originally described in the transformation of plant tissue (Klein, T.M. *et al.* (1987) *Nature 327*:70; Christou, P. *et al.* (1990) *Trends Biotechnol. 6*:145) but also applicable to mammalian tissues *in vivo*, *ex vivo* or *in vitro*

(Yang, N.-S., *et al.* (1990) *Proc. Natl. Acad. Sci. USA 87*:9568; Williams, R.S. *et al.* (1991) *Proc. Natl. Acad. Sci. USA 88*:2726; Zelenin, A.V. *et al.* (1991) *FEBS Lett. 280*:94; Zelenin, A.V. *et al.* (1989) *FEBS Lett. 244*:65; Johnston, S.A. *et al.* (1991) *In Vitro Cell. Dev. Biol. 27*:11). Furthermore, electroporation, a well-known means to transfer genes into cell *in vitro*, can be used to transfer DNA molecules according to the present invention to tissues *in vivo* (Titomirov, A.V. *et al.* (1991) *Biochim. Biophys. Acta 1088*:131).

Gene transfer can also be achieved using "carrier mediated gene transfer" (Wu, C.H. *et al.* (1989) *J. Biol. Chem. 264*:16985; Wu, G.Y. *et al.* (1988) *J. Biol. Chem. 263*:14621; Soriano, P. *et al.* (1983) *Proc. Natl. Acad. Sci. USA 80*:7128; Wang, C-Y. *et al.* (1982) *Proc. Natl. Acad. Sci. USA 84*:7851; Wilson, J.M. *et al.* (1992) *J. Biol. Chem. 267*:963). Preferred carriers are targeted liposomes (Nicolau, C. *et al.* (1983) *Proc. Natl. Acad. Sci. USA 80*:1068; Soriano *et al.*, *supra*) such as immunoliposomes, which can incorporate acylated monoclonal antibodies into the lipid bilayer (Wang *et al.*, *supra*), or polycations such as asialoglycoprotein/polylysine (Wu *et al.*, 1989, *supra*).

In general, improved efficiency of gene transfer is attained by the use of promoter enhancer elements in the plasmid DNA constructs (Philip, R., *et al.* (1993) *J. Biol. Chem.* 268:16087-16090).

The disclosure provided herein focuses on the gp120-derived amino acid sequence that is present in the final fIg product. The nucleotide sequences encoding the desired peptide epitopes are not specifically listed here but are evident to those skilled in the art. First, the full native sequences for HIV gpo120 (consensus for each subtype as well as individual viral isolates reported to date) are provided in the Compendium cited above. One skilled in the art will know how to utilize alternate coding sequences for expressing the desired native or synthetic gp120 peptides which are to be included in the tolerogenic fIg. Any nucleotide sequence which encodes a chosen peptide epitope or series of epitopes may be used. Distinct gp120 epitopes may be combined in any order or combination provided that the coding nucleic acids

provide an in-frame sequence both with respect to the gp120 epitopes and with respect to the Ig H gene utilized to construct the fIg.

Uses of the Invention

Treatment of an individual infected with HIV using the tolerogenic fIg of this invention comprises parenterally administering a single or multiple doses of the fIg to a subject, preferably a human. The fIg is preferably an isologous Ig, that is, of the same species as the subject. A most preferred fIg is fusion IgG molecule. An effective tolerogenic dose is a function of the size and number of particular HIV gp120 epitopes included in a particular fIg construct, the patient and his clinical status, and can vary from about 0.01 mg/kg body weight to about 1 g/kg body weight. A subject can be given this amount in a single dose or in multiple repeated doses.

Doses of hematopoietic cells or B cells expressing the fIg are preferably administered at a dose between about 10^6 and 10^{10} cells on one or several occasions.

The route of administration may include intravenous (iv) , subcutaneous (SC), intramuscular, intrapulmonary, intraperitoneal or other known routes. The preferred route for administration of fIg proteins or cells for tolerogenesis is by iv injection.

The fIg of this invention may be advantageously utilized in combination with other therapeutic agents useful in the treatment or prevention of HIV disease, including prophylactic or therapeutic vaccine preparations, antiviral chemotherapeutic agents, immune response modulators including cytokines and hematopoietic growth factors, protective antibody reagents, *etc.*

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

EXAMPLE I

Epitope-Specific Tolerance Induction by Gene Transfer of an Engineered Peptide-Immunoglbulin Fusion Protein

In this study, the present inventors took advantage of the IgG molecule as a tolerogenic carrier, and created an engineered tolerogen with a grafted epitope at the

N-terminus of an IgG heavy chain. This engineered IgG was recognized by the immune system in a tolerogenic manner. The model epitope chosen for this initial analysis is the well-characterized class-II MHC-restricted peptide sequence from the cI λ repressor protein (p1-102), residues 12-26. This peptide contains both a B- and T-cell epitope, and is the immunodominant determinant in H-2^d mice immunized with the entire protein (26-30). It was thus possible to measure tolerance induction to a single determinant at both the B-cell and T-cell levels. Furthermore, tolerogenic Ig-peptide constructs could be expressed in adoptively-transferred hematopoietic tissue for the permanent modulation of epitope-specific immune responses in mature adults. These studies show that the model fIg, 12-26-IgG, is an efficient tolerogen in adult animals and serves as the basis for expansion of this approach to other epitopes of clinical utility as described below.

MATERIALS AND METHODS

Mice. Male and female BALB/cByJ (H-2^d) and CAF1 (H-$2^{d/a}$) mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and were used at 6-10 weeks of age.

Medium: RPMI 1640 medium (GIBCO-BRL, Gaithersburg, MD) was supplemented with 5% FCS (Hyclone, Logan, UT), 2-ME, L-glutamine, penicillin, streptomycin, MEM nonessential amino acids, and sodium pyruvate.

Antibodies: Hybridoma B3.11, which produces a monoclonal IgG_1 specific for the 12-26 peptide was a kind gift of Drs. Tom Briner and Malcolm Gefter (Immulogic, Waltham, MA). B3.11 was affinity purified with goat anti-mouse IgG sepharose columns and biotinylated, or used as a neat culture supernatant. All alkaline-phosphatase (AP)-conjugated reagents were purchased from Southern Biotechnology Assoc. (Birmingham, AL).

Synthetic peptide: The 12-26 15-mer LEDARRLKAIYEKKK (SEQ ID NO:112) was prepared with a solid-phase method and purified to >92% homogeneity using standard HPLC methods. Peptide was conjugated to bovine albumin serum (BSA) rabbit gamma globulin (RGG), or keyhole limpet hemocyanin (KLH) as described (Roy, S. *et al.* (1989) *Science. 244*:575-575).

Oligonucleotides: The following complementary synthetic oligonucleotides encoding the 12-26 sequence were designed with BamHI/ClaI restriction ends, phosphorylated with T4 kinase and ATP, and cloned into the hypervariable region of flagellin construct pPX1647:

DWS1: (SEQ ID NO:195)
5'-CGA TCT GGA GGA CGC GCG GCG GCT GAA GGC GAT ATA CGA GAA GAA GAA GG-3'
DWS2: (SEQ ID NO:196)
5'-GAT CCC TTC TTC TTC TCG TA T ATC GCC TTC AGC CGC CGC GCG TCC TCC AGA T-3'
PCR primers were also designed to amplify a modified 12-26 sequence from the chimeric 12-26-flagellin construct. This sequence includes 5' FR1 V_H sequence and PstI restriction sites at each flanking ends:

Ig-one: 5'-TGATCTACTGCAGCTGGAGGACGCGCGGCG G-3' (SEQ ID NO:197)
Ig-two:
5'- CGACCTCCTGCAGTTGGACCTGCTTCTTCTTCTCGTATAT-3' SEQ ID NO:198)

ELISA: To determine the specificity of binding of our peptide-specific mAb B3.11 to 12-26-fusion proteins, competitive inhibition ELISA's were conducted as follows: biotinylated B3.11 was incubated 1:1 (vol/vol) with decreasing amounts of inhibitor in ELISA binding buffer (0.25% BSA, 0.05% Tween 20 in saline). Mixtures were then incubated on peptide-coated (10 μg/ml) ELISA plates (Immulon 4 Dynatech), and subsequently streptavidin-AP was added as a secondary reagent. Percent inhibition of binding (A_{405}) was calculated as: [(average binding of antibody alone minus average binding of antibody incubated in presence of inhibitor)/average binding of antibody alone] x100. ELISA determinations of serum peptide-specific IgG responses were done by coating ELISA plates with 50 μg/ml synthetic peptide. Antigen-coated plates were blocked with 1% gelatin/0.05% Tween 20 buffer, and duplicate serial dilutions of serum were incubated and probed with goat anti-mouse IgG isotype-specific secondary reagents. Titers are expressed as the geometric mean of the reciprocal dilution required to bring A_{490} readings to prebleed levels or <0.08 O.D.

Protein Engineering Design:

Preparation of murine H chain IgG_1 construct encoding the 12-26 sequence at the N-terminus

Our strategy for inserting a foreign peptide sequence at the N-terminus of an IgG H chain is similar to what has been described (Hebell, T. *et al.* (1991) *Science 254*:102-105; Dal Porto, J. *et al.* (1993) *Proc. Natl. Acad. Sci. USA 90*:6671-6675).

Plasmid pSNR (Ballard, D.W. *et al.* (1986) *Proc. Natl. Acad. Sci. USA. 83*:9626-9630), which contains neo and amp resistance genes, as well as the full genomic sequence for a IgG_1^b H chain specific for the NP hapten, was obtained from Dr. Douglas Fearon (Cambridge University) and modified. A modified 12-26 sequence was created via PCR amplification of this sequence from the chimeric flagellin construct A29 (described in WO95/21926) utilizing PCR primers "Ig-one" and "Ig-two". The modified 12-26 sequence was subcloned into the V_H site of pSNR and recombinant clones were analyzed for proper orientation and Taq polymerase mutational errors by double-stranded DNA sequencing methods (USB Sequenase 2.0 kit).

Expression, purification, and quantitation of transfected IgG:

Construct pQ3.EZ1 (Q3), as well as the control pSNR IgG_1 construct (P6) were electroporated into J558L myeloma cells (which produce only a λ light chain) as described by Hebell *et al., supra,* Ballard *et al., supra,* and Dal Porto *et al., supra.* Stably transfected clones were isolated in 1 mg/ml G418 (GIBCO-BRL), subcloned, and transfected IgG's from selected clones were purified from bulk supernatants or ascites with anti-mouse IgG-Sepharose or protein G columns. Since the original H chain binds with high affinity to the NIP (5-iodo-4-hydroxy-3-nitrophenylacetyl) hapten, purified or serum transfectoma IgG was quantitated using a modified NIP-gelatin binding ELISA , using anti-mouse IgG_1-AP as a secondary reagent.

In vitro and *in vivo* tolerance induction and immunization protocols:

Peptide-specific tolerance induction in adult recipients was accomplished by intravenous ("iv") injection (in the lateral tail vein) of either 1 mg purified, deaggregated, chimeric (Q3) or control IgG (P6) diluted in saline, or by 3 repeated

injections of mitomycin C-treated (50 μg/ml, SIGMA) P6- or Q3-secreting transfectomas. For measurement of humoral immune responses 10 days following iv tolerization, animals were immunized subcutaneously ("SC") at the base of the tail and intraperitoneally ("ip"), with 50 μg synthetic 12-26 peptide and 25 μg hen egg lysozyme (HEL, SIGMA) emulsified in Freund's complete adjuvant (CFA, SIGMA). Mice received an additional antigenic boost of 50 μg peptide and 25 μg HEL injected ip in saline 2 weeks after initial priming. Mice were bled to assess serum anti-peptide antibody responses 8 days after this boost. Splenic memory T cell ("tertiary") responses were analyzed in culture 8 weeks following secondary boosts. Splenic T cells were enriched by panning on anti-Ig coated plates, and restimulated ($3x10^6$/ml) with dilutions of peptide and irradiated splenic APC (2500 rads, 10^6/ml). For analysis of secondary (LN) responses following iv tolerization, animals were immunized in hind footpads with 20 μg peptide emulsified in CFA, and draining popliteal LNs were harvested 9 days later and restimulated in culture with dilutions of peptide and 50 μg/ml purified protein derivative (PPD, Connaught, Swiftwater, PA). IL-2 and IL-4 secreted into the medium were determined from culture supernatants at 24 and 48 hours, respectively, in LN or splenic T-cell cultures using recombinant cytokines as standards.

In vitro B-cell tolerance induction experiments were done on enriched splenic B cells essentially as described by before (Waldschmidt, T.J. *et al.* (1983) *J. Immunol. 131*:2204-2209; Phillips, N.E. *et al.,* (1983) *J. Immunol. 130*:602-606; Warner, G.L. *et al.,* (1991) *J. Immunol. 146*:2185-2191). Supernatants from 3-4 day cultures were assayed for IgM production by ELISA by coating wells with peptide- or FITC-BSA conjugates, and probing with goat anti-mouse IgM-AP. Results represent experiments repeated 2-3 times; individual points represent the arithmetic mean of triplicate or quadruplicate values with standard deviations generally less than 15% (omitted for clarity).

RESULTS

Preparation and characterization of a murine IgG_1 self-carrier containing the λ cI repressor 12-26 peptide at the V_H N-terminus

The 12-26-IgG construct was prepared by modifying plasmid pSNR, which contains the genomic sequence encoding a murine IgG_1^b H chain. Isologous IgG_1 was chosen because of its documented activity as a tolerogenic "carrier" of potency equal to IgG_2, and greater than other Ig isotypes or other serum proteins. We chose to insert a foreign epitope at the N-terminus of the V_H region (Figure 5A), because insertions at this location have been shown not to alter normal immunoglobulin folding and structure (Hebell *et al., supra;* DaPorta *et al., supra*). Analysis of transfected, purified chimeric 12-26-IgG (Q3) or control pSNR IgG (P6) by SDS-PAGE showed that H chains can successfully pair with J558L light chains (L). The chimeric H chain (Q3) containing the additional 12-26 sequence was about 1.8 kDa larger than the control IgG (P6).

Purified transfected IgGs expressed the 12-26 epitope as shown by western blotting and ELISA utilizing peptide-specific mAb B3.11 (Figure 5B). Furthermore, in competitive inhibition ELISA, chimeric 12-26-IgG effectively competed with free synthetic peptide or a chemical conjugate of 12-26 with rabbit IgG for binding to mAb B3.11. These results suggest that the inserted peptide is recognized efficiently by epitope-specific antibodies/B cells on the exterior surface of the recombinant IgG, without significantly perturbing H chain tertiary structure.

Additionally, the recombinant 12-26-IgG chimera is immunogenic and capable of priming 12-26-specific T and B cells *in vivo*. Mice immunized with Q3 emulsified in CFA were able to prime 12-26-specific T cells comparable to the response elicited with synthetic peptide. *In vitro* restimulation of LN cultures with synthetic peptide resulted in T-cell proliferation as well as IL-2 and IL-4 production in peptide- and Q3-primed, but not P6-primed LN cells. Immunization also led to a high serum anti-12-26 IgG antibody titer detectable by peptide-specific ELISA. 12-26-IgG stimulated IL-2 production in an $I\text{-}A^d$-restricted 12-26 specific T-cell hybridoma (9C127). These results suggest that the confirmation of the inserted foreign epitope is not only

recognized by specific antibodies, but the peptide (or one like it) can also be processed and presented to T cells in a physiologically relevant manner by APC even in the context of a self-IgG scaffold.

In vivo induction of peptide-specific immune self tolerance with soluble purified engineered 12-26-IgG.

To test the efficacy of genetically engineered tolerogens, we analyzed both humoral and cellular immune responsiveness after iv administration of high doses of soluble, deaggregated 12-26-IgG fusion Ig. Mice were injected with 1 mg of either chimeric Q3 or control P6 immunoglobulins, and challenged 10 days later with a mixture of 12-26 peptide and HEL (as a specificity control) emulsified 1:1 in CFA. Secondary humoral immune responses were analyzed one week after an additional boost. Figure 6 shows that mice receiving pretreatments of Q3, but not control P6, were dramatically unresponsive to peptide challenge as assessed by ELISA of anti-peptide IgG, whereas control anti-HEL antibody titers were unaffected. Although the predominant Ig isotype in this anti-peptide response in Balb/c mice is IgG_1, antibodies of all isotypes including IgG_{2b} were consistently diminished by the tolerogenic treatment with 12-26-IgG (Figure 6).

To test the potential of inducing unresponsiveness with peptide-Ig-transfected cells as a model for gene-therapy-based tolerogenesis, Balb/c mice received 3 (consecutive weekly iv injections of transfectomas secreting Q3 (or P6 control IgG); the cells had first been treated with mitomycin C. This protocol resulted in transient appearance in serum of the transfected IgG's at levels reaching at least 10-500 ng/ml (assessed by NIP-gelatin ELISA). This type of treatment resulted in diminution of peptide-specific humoral immune responses as well as reduction of LN cell proliferative responses.

Since unresponsiveness as measured by serum antibodies may results from tolerance of B-cells, T-cells or both, the cellular basis of the observed tolerance was analyzed by measuring T-helper (Th) cytokine responses 8 weeks after immunogenic challenge. Restimulation of splenic memory T cells (Figure 7) revealed that both Th1-type (IL-2) and Th2-type (IL-4) responses were absent in tolerized mice, a result

consistent with the observed lack of anti-peptide IgG_{2b} and IgG_1 antibodies(Figure 6), which are dependent on these Th cell subsets. The T cell response to peptide was diminished in 12-26-IgG pretreated animals when measured as short-term LN restimulation assays . Mice tolerized with 1 mg of 12-26-IgG 10 days previous to peptide challenge had reduced LN IL-2 responses, but unaffected recall proliferative responses to the antigen PPD compared to control P6-injected animals. These results indicate efficient induction of Th cell tolerance to the immunodominant peptide which results in an inability to prime any subset of T cell response to the peptide.

Thus, a foreign immunogenic peptide genetically engrafted into an Ig scaffold can be very efficiently presented to the immune system in a tolerogenic manner when administered by the appropriate route and method. Thus pretreatment with peptide-Ig chimeras delivered either as single high doses or via slow release by transfected autologous B cells have utility in achieving efficient epitope-specific manipulation of undesired T-cell responses.

Analysis of a novel 12-26-flagellin immunogen for testing the efficacy of 12-26-IgG on B-cell tolerance.

To test the efficacy of the fIg 12-26-IgG as a B-cell tolerogen, it was necessary to challenge B cells with an immunogenic, T-independent form of the 12-26 epitope. Since polymerized flagellin is a well-characterized T-independent antigen, we constructed a 12-26 flagellin fusion protein with a strategy previously described (Newton *et al., supra*). Western blotting and ELISA analyses of purified WT (pPX) and 12-26-flagellin (A29) showed that although flagellin epitopes are readily expressed in both recombinant flagellins, the inserted epitope was detectable only in chimeric flagellin A29. Polymerized 12-26-flagellin stimulated splenic B cells to secrete anti-12-26 IgM antibodies. The stimulatory effect was comparable to that of the polyclonal B cell mitogen, bacterial lipopolysaccharide (LPS). A concentration of 0.1 μg/ml was found to be minimally mitogenic (as assessed by anti-fluorescein [FITC] IgM ELISA's) and used for subsequent experiments. These results broaden the context in which the inserted epitope can be recognized: IgG and the polymerized flagellin molecule. In the latter context, the epitope readily stimulate B cells to produce epitope-specific IgM antibodies.

We also tested the ability of 12-26-IgG to induce specific B-cell unresponsiveness. Enriched B cell populations were incubated *in vitro* with various doses of Q3 or P6 control IgG's, washed, and then cultured with either mitogenic LPS or 12-26-flagellin. Alternatively, BALB/c mice were injected iv with 1 mg of each protein, and splenic B cells were harvested and challenged *in vitro* 10 days later. Supernatants from 3 day cultures were assayed for 12-26-specific IgM antibody or anti-FITC antibody as a specificity control. Pretreatment with Q3, but not P6, either *in vitro* or *in vivo,* markedly suppressed the anti-12-26 IgM response, whereas anti-FITC control IgM responses were unaffected. Thus, in addition to inducing potent Th tolerance, the flg construct is independently can induce epitope-specific unresponsiveness in B cells. The magnitude of B cell tolerance was more modest *in vivo* than T cell tolerance, possibly reflecting either a requirement for higher epitope valency (the flg provides only a bivalent epitope, one on each arm of the H chain), or a higher dose requirement. A reduction of antibody responsiveness of similar magnitude was observed after adoptive transfer of *in vivo*-tolerized B cells, admixed with nontolerized naive T cells, into secondary immunodeficient recipients which were then challenged with the peptide in CFA.

DISCUSSION

The development and maintenance of the unresponsive state in newly emerging lymphocytes is a lifelong process requiring the persistence of antigen. Exposure of mature B and T cells to antigen in an adult immune system may lead to either activation or tolerance depending on the route and method of exposure, as well as the availability of costimulatory signals from specialized APC. Since a major goal in clinical therapy in a variety of conditions (*e.g.*, infection, autoimmunity, allergy, transplantation) is the induction of specific immune unresponsiveness in adult mature lymphocytes, a variety of approaches have exploited these pathways of exposure. Of these approaches, experimental tolerance induction with gamma-globulin carriers has been most extensively described. IV administration of soluble, deaggregated IgG's in the absence of adjuvants, induces both antigen-specific B-cell and T-cell tolerance even in the absence of a thymic environment. Mechanisms of specific clonal

anergy/inactivation and deletion have been implicated in this type of experimental model.

In the foregoing studies, the present inventors described for the first time, the tolerogenic capability of an engineered self-IgG expressing a model class II MHC-restricted immunodominant peptide. This novel epitope, deliberately expressed at the N-terminus of an IgG heavy chain construct, was tolerogenic *in vivo* and *in vitro*. Conceptually similar approaches have been utilized to express immunogenic (rather than tolerogenic) malarial or viral peptides in the CDR3 loop of Ig H chains for the induction of enhanced anti-peptide immune responses, as described above.

As with other such epitopes, the 12-26-IgG protein could act as an efficient immunogen when administered in an immunogenic manner (*i.e.*, emulsified in CFA). Zaghouani *et al.*, 1993, *supra* showed that T-cell activation (for a class II-restricted epitope) was enhanced 100-1000 fold when the epitope was part of an Ig-chimera, presented *in vitro* by stimulatory dendritic cells as APC. The present results similarly show that an approximately 100-fold lower molar quantity of 12-26-IgG (as compared to free peptide) stimulated similar numbers of peptide-specific LN T cells from immunized mice.

The increased efficacy of the fIg's of the present invention, both as immune activators and as tolerance inducers, may indicate that common pathways are utilized. The increased efficacy may directly result from (a) an increased half-life and (b) an Fc-receptor mediated uptake of the "carrier" portion of the Ig molecule (Stockinger, B. (1992) *Eur. J. Immunol. 22*:1271-1278) leading to improved presentation of the grafted foreign epitope(s). In the absence of adjuvants (which act in part by mobilizing APC having efficient costimulatory capability), high doses of soluble, deaggregated serum protein may be preferentially taken up by "non-professional" APC, such as resting B cells, via the process of Fc receptor-mediated endocytosis or phagocytosis, and subsequently presented by these "non-professional" APC (Parker, D.C. *et al.* (1991) *FASEB J. 5*:2777-2784; Eynon, E.E. *et al.* (1992) *J. Exp. Med. 175*:131-138; Fuchs, E.J. *et al.* (1992) *Science 258*:1156-1159). Furthermore, IgG carriers can induce efficient B-cell unresponsiveness by mechanisms involving the

crosslinking of surface IgM to Fc receptors. One or more of the above mechanisms may be responsible for the enhanced tolerogenic efficiency of Ig carriers. In contrast, the mere iv injection of soluble, deaggregated peptides can suffice to induce effective Th cell tolerance (Scherer, M.T. *et al.* (1989) *Cold Spring Harbor Symp. Quant. Biol. 54*:497-504), but is insufficient to induce specific B cell unresponsiveness.

The present inventors findings are summarized as follows. A fIg, specifically the 12-26-IgG fusion protein, can present an epitope in a tolerogenic fashion and induce both B- and T-cell tolerance. A convenient property of this epitope allows simultaneous study of both cellular and humoral immune responses to a single immunodominant peptide. The 12-26 peptide can induce a vigorous antibody response which is predominantly of the IgG_1 isotype, and can prime Th cells of both the Th1 and Th2 phenotype. Tolerance induction with 12-26-IgG was globally effective in suppressing every type of immune response which can be elicited by this immunodominant peptide.

The inventors have therefore provided a powerful approach to determining the efficacy of inducing specific unresponsiveness to a defined antigens, particularly peptide antigens, for the modulation of undesired immune responses. The present approach has advantages of that inserting heterologous epitopes into the H chain CDR3 because the N-terminus insertion does not restrict the size of the epitope or epitopes fused to the tolerogenic IgG carrier. Therefore, not only short peptides, but also larger, more complex foreign antigens may be fused in an fIg construct for tolerogenic presentation.

Finally, because this approach provides what may be envisioned as a genetically transmissible "hapten-carrier" complex, these tolerogenic fIgs when expressed as a transgene-transferred into hematopoietic tissues or cells, can be used to both induce and maintain tolerance for the long term. Such studies are reported in Example III, below. Recipients of BM stem cells which have been transduced with a retroviral vector for the long-term expression of fIg cDNA constructs. The application of most immediate interest for the present invention is the use of this

approach to block and ineffective and potentially harmful antibody responses which occur during HIV infection (Clerici, M. *et al.*, (1993) *Immunol. Today 14*:107-110).

EXAMPLE II

Tolerance to HIV gp120 Epitopes from the C5 Region: Detection

The study described above using a phage λ epitope was extended to two gp120 epitopes:

(1) a C5 peptide KYKVVKIEPLGVAPTKAKRRVVQREKR (SEQ ID NO:199)

positions 485-511 of gp120 from the BH10 strain (see Figure 3) which is cross-reactive with HLA-C monomorphic determinants (DeSantis, C. *et al.* (1993) *J. Infectious Dis. 168*:1396; Palker, T.J. *et al.* (1987) *Proc. Nat'l Acad. Sci. USA 84*:2479; and

(2) a C1 immunodominant peptide (such as residues 90-120 of the BH10 isolate, above, that contains distinct B- and T-cell epitopes (Abacioglu *et al.* (*supra*).

Many antibody responses to HIV can be non-protective, and can enhance viral uptake by monocytes or promote T-cell apoptosis (Finkel *et al.*, *supra*; Banda *et al.*, *supra*; Kliks, S.C. *et al.* (1996) *Proc. Nat'l Acad. Sci. USA 90*:11518. Initially, the inventors focused on defining peptides smaller than the original 35-mer in C5 defined by Beretta and colleagues (DeSantis, C. *et al.* (1993) *J. Infectious Dis. 168*:1396. Using mAbs (Robinson, W.E. Jr. *et al.* (1990) *Proc. Nat. Acad. Sci. USA 87*:3185, peptides were examined that contain either the KYK or KAKRR (SEQ ID NO:200)motifs that have been defined for HLA cross-reactivity. At least two 15-18-mers were identified by an ELISA inhibition assay.

Results discussed in Example I, above, indicated that, in contrast to free peptide, λ 12-26-IgG was tolerogenic for B cells as a bivalent molecule, although the mechanism of this unresponsiveness was not determined.

Design of shortened peptides containing major C5 epitopes:

As a model epitope, the inventors initially chose the C-terminal peptide KYKVVKIEPLGVAPTKAKRRVVQREKR (SEQ ID NO:199)

(residues 485-51 in the BH10 variant (Figure 3) and which ; corresponds approximately to positions 455-481 of the consensus sequence in Figure 1). This is in the conserved C5 C-terminal region of gp120. This peptide contains the B-cell epitope consisting of the KYK--------KAKRR (SEQ ID NO:200)motifs that are recognized by the M38 murine mAb (DeSantis *et al.*, *supra;* Palker *et al.*, *supra*). The epitope recognized by M38 has been noted to be KYKVVKEIPLGVAPTKAKRR of SEQ ID NO:199. MAb M38 also binds to the C-terminus of gp120, in a gp41 binding region. M38 also reacts with a common motif in the HLA-C heavy chain a1 region (KYKRQAQADRVNLRKLR; SEQ ID NO:201) that is mimicked in this C5 peptide. HIV-infected individuals have HLA class I-gp120 cross-reactive antibodies.

The inventors first established that a 35-mer containing this M38-defined epitope was tolerogenic *in vivo* when chemically coupled to heterologous rabbit IgG. Since the C5 peptide was relatively large and not readily available. Shorter peptides containing the KYK and KAKRR sequences with different spacer residues and with a C-terminal cysteine for more controlled coupling to IgG carriers can be designed. Although the residues between the two M38 epitopes (IEPLGVAPT; SEQ ID NO:202) are not recognized in seropositive individuals (Scott, D.W. *et al.* (1993) *Adv. in Molec. and Cell. Immunol. 1*:119, it was important to determine the contribution of these amino acids to the epitope conformation. The mAbs described in Robinson *et al.*, *supra*, were used to analyze reactivity to these new peptides as well as the requirement for the intervening sequences. The peptides designed were:

1. AAKYKGGGGGKAKRRGGC (SEQ ID NO:203)
2. AAKYKGGGPTKAKRRGGC (SEQ ID NO:204)
3. AAKYKGVAPTKAKRRGGC (SEQ ID NO:205)

Control peptides (for example, available from the National Institute of Allergy and Infectious Diseases) encompassed the KYK, KAKRR (SEQ ID NO:200) motifs or the entire IEPTGVAPTKAKRR (SEQ ID NO:206)sequence recognized by the human mAbs. Using a competitive ELISA assay, the inventors found that peptide #3, above ("P3"), was similar in activity to the full sequence and that peptides containing only the KYK motif were noninhibitory, as expected, with these human anti-C5

mAbs. Treatment with P3-HGG induced unresponsiveness for an anti-P3 response (and an anti-HGG response). These results suggest that VAPT motif, while not immunoreactive, contributes to the structural integrity of this epitope. Peptide 3 (P3) contains both motifs that have been reported to be recognized by M38 and are important in anti-HLA-C recognition, for which tolerance induction is one goal. Importantly, these results mean that shorter peptides still express B-cell epitopes.

It is expected that anti-C1 and V3 loop antibody responses would not be affected by the above peptides because the stimulus is a polyclonal mitogen. It is expected that anti-HLA crossreactivity will be eliminated if KYK-specific unresponsiveness is induced. In order to achieve tolerance to C1 region or V3 loop epitopes, the present invention would require that the flg include one or more epitopes from these regions.

EXAMPLE III

Resting and Activated B Lymphocytes Expressing flg are Tolerogenic Vehicles

Since antigen-presenting B-lymphocytes are known to either augment or downregulate T-cell dependent immunity , it should be possible to modulate the immune response to a selected antigen (such as an autoantigen, a viral antigen or a tumor antigen) via gene-transfer of exogenous genes and constitutive expression *in vivo* by autologous APC. Such an approach would be advantageous for the induction of unresponsiveness, since tolerance to foreign antigens could be maintained indefinitely *in vivo*, especially if gene-transfer into long-lived lymphoid progenitors is achieved.

Previous models have led to apparently divergent results and have shown that B cells can be either essential (Ron, Y. *et al.* (1981) *Eur. J. Immunol. 11*:964-968; Janeway, C.J. *et al.* (1987) *J. Immunol. 138*:1051-1055; Constant, S. *et al.* (1995) *J. Immunol. 155*:3734-3741; Morris, S.C. *et al.* (1994) *J. Immunol. 152*:3777-3785) or nonessential (Sunshine, G.H. *et al.* (1991) *J. Exp. Med. 174*:1653-1656; Ronchese, F. *et al.* (1993) *J. Exp. Med. 177*:679-690; Epstein, M.M *et al.* (1995) *J. Exp. Med. 182*:915-922) for T cell priming, and can be critical for either activating or tolerizing (Eynon, E.E. *et al.* (1991) *Transplant. Proc. 23*:729-730; Eynon, E.E. *et al.* (1992) *J.*

Exp. Med. 175:131-138; Fuchs, E.J. *et al.* (1992) *Science. 258*:1156-1159; Buhlmann, J.E. *et al.* (1995) *Immunity. 2*:645-653) naive T cells, and even previously activated T-cell clones (Gilbert, K.M. *et al.* (1994) *J. Exp. Med. 179*:249-258). The state of activation of the collaborating B cells and T cells as well as the antigen-specificity for the interactions have appeared to be important for the different outcomes.

To analyze the ability of antigen-presenting B cell to serve as a modulator of the immune response, the present inventors generated a unique transgenic mouse system (see Example I) in which a foreign class II-restricted immunodominant epitope is expressed as a self antigen specifically in the B cell compartment. The foreign epitope, residues 12-26 from λ cl repressor protein was grafted in-frame at the N-terminus of a murine IgG_1 heavy chain and is made endogenously as a transgene in the B-lymphocyte lineage. The tolerogenic capabilities of this soluble engineered immunoglobulin in immunocompetent adult mice is described above. The present study describes the tolerogenic nature of transgenic hematopoietic tissue expressing such a flg molecule. This approach takes advantage of the efficiency of the immunoglobulin secretory and endocytic pathways to synthesize and present an exogenous "neo" self-peptide, and provides a model for inducing peripheral tolerance to undesirable humoral and cellular immune responses using gene therapy strategies.

I. Materials and Methods

Mice and Reagents. Male and female B6D2 ($H\text{-}2^{b/d}$) and BALB/cByJ ($H\text{-}2^{d}$) mice were purchased from the Jackson Laboratories (Bar Harbor, ME) at 3-8 weeks of age, and housed in pathogen-free, microisolater cages. RPMI 1640 medium (GIBCO-BRL, Gaithersburg, MD) was supplemented with either heat-inactivated 5% FCS (Hyclone, Logan, UT), or heat-inactivated 0.5% autologous mouse serum (Jackson Immunochemicals), 2-ME, L-glutamine, penicillin, streptomycin, MEM nonessential amino acids, and sodium pyruvate. Hybridoma B3.11, which produces an IgG_1 specific for the 12-26 peptide was from Drs. T. Briner and M.Gefter (Immulogic Corp., Waltham, MA), and was originally derived by fusion with splenocytes from peptide-immunized BALB/c mice. Monoclonal antibody (mAb) B3.11 was affinity purified from bulk-cultured supernatants with goat anti-mouse IgG sepharose columns

and biotinylated. All alkaline-phosphatase (AP)-conjugated secondary reagents were purchased from Southern Biotechnology Assoc. (Birmingham, AL). The 12-26 15-mer LEDARRLKAIYEKKK (SEQ ID NO:112), or an N-terminal cysteine-modified 16-mer was prepared with a solid-phase method and purified to >92-95% homogeneity using standard HPLC methods. The cysteine-modified 12-26 peptide was covalently conjugated to hen egg white lysozyme (HEL) with Sulfo-MBS (Pierce, Rockford, Illinois), a sulfhydryl-specific crosslinking reagent.

Generation of Transgenic (Tg) Mice. The preparation of a chimeric murine IgG_1^b H chain construct, specific for the NP hapten and engineered to express the 12-26 peptide at the N-terminus, is described in detail above. The entire ~10 kb genomic construct containing the original endogenous immunoglobulin promoter, enhancer, and polyadenylation sequences was shuttled into pBluescript KS+/- (Stratagene, La Jolla, CA) and excised as a XhoI/NotI fragment. The linearized transgene was purified over a continuous 10-40% (wt/vol) sucrose gradient and dialyzed against 5 mM Tris-HCl/0.15 mM EDTA (ph 7.5). Tg mice were derived by pronuclear injection of fertilized B6D2 eggs, and implantation into pseudopregnant females as described by Hogan *et al.*(Hogan, B. *et al.* (1986) *Manipulating the Mouse Embryo*: *A Laboratory Manual*. Cold Spring Harbor Lab. Press, Plainview, N.Y. pp. 81-141, incorporated by reference). Three original Tg founders were identified by genomic Southern blotting of tail DNA with a ^{32}P-labeled probe containing 3 cloned, tandem copies of the 12-26 cDNA sequence. Two of these founders (Line 5 and Line 17) were selected for further analysis, bred onto the BALB/c background for at least 5-10 generations, and confirmed for $H\text{-}2^d$ homozygosity via RFLP Southern blot analysis before use in BALB/c adoptive transfer experiments. Lines 5 and 17 were also rederived by Cesarean section (Taconic Labs) and thereafter housed in sterilized microisolater units at the Holland Laboratory to ensure healthy microorganism-free strains of Tg mice. Tg offspring obtained via BALB/c matings were, in general, heterozygous for their transgene and distinguished from their nontransgenic (NTg) littermates by either 12-26 sequence Southern blotting of tail DNA, or serum NIP-binding IgG_1 ELISA.

Preparation of Bone Marrow Chimeras. Eight week-old BALB/c recipients were sublethally irradiated (650 rads) with a ^{137}Cs source and injected iv with 10^7 cells consisting of a 1:1 pooled mixture of non-Tg/Line 17 Tg bone marrow (BM) cells that had been depleted of erythrocytes. Control mice were injected with NTg littermate BM cells(after 650 rads) or saline only (with no irradiation). All Tg/NTg donor BM was completely sex-matched and syngeneic with BALB/c recipients. Adoptively-transferred mice were rested for 7-8 weeks before immunization studies.

Preparation of Lymphoid Cells for Tolerance Induction. 12-26 peptide-specific tolerance induction in normal adult (6-10 week old) BALB/c mice was accomplished by iv injection of Line 17 Tg hematopoietic tissue. Unconditioned recipients were generally injected with 2-4 x 10^7 cells from preparations of purified resting B cells, LPS-activated B cell blasts, unfractionated splenocytes, or crude BM cells from Tg or control NTg donors. Ten days following such injections, recipients were immunized with antigens SC as described below.

Bone marrow (from both femurs and tibiae) or spleen tissue was prepared in serum-free complete RPMI and depleted of erythrocytes. Splenic B cells were obtained by depleting splenocytes of T cells by treatment with anti-T cell cocktail plus baby rabbit complement. Resting B cells were harvested by further fractionation on Percoll gradients and collecting the 60-70% layers as previously described (29). For preparation of activated B cell populations, purified B cells ($4x10^6$/ml) were incubated for 48 hrs in complete RPMI (5% FCS) in the presence of 50 μg/ml LPS (Sigma, St. Louis, MO), and washed 3 times before further use. For chemical fixation, purified B cells were treated with carbodiimide (ECDI, Sigma) by incubating 10^8 cells in 0.5 ml of 75 mM ECDI (in saline) for 1 hour, on ice. All cells were washed extensively prior to iv injection.

Measurement of Peptide-Specific Cellular and Humoral Immune Responses. All protocols are essentially as described previously (Lai, M-Z *et al.* (1987) *J. Immunol. 139*:3973-3980; Scherer, M.T. *et al.* (1989) *Cold Spring Harbor Symp. Quant. Biol. 54*:497-504; Soloway, P. *et al.* (1991) *J. Exp. Med. 174*:847-858). Ten days following iv tolerization, animals were immunized to induce cellular or humoral

immune responses. For measurement of humoral immunity, animals were injected SC at the base of the tail with 50 μg synthetic 12-26 peptide emulsified 1:1 in complete Freund's adjuvant (CFA). In some experiments, animals were also injected with 20 μg hen egg lysozyme (HEL) in CFA, intraperitoneally (ip). Two weeks later, mice received an additional antigenic boost of 50 μg peptide and 10 μg HEL in saline, injected ip. Antibody titers were determined from serum obtained 8 days after secondary boosts. Splenic memory T cell responses were measured *in vitro* 6-8 weeks following these secondary challenges.

For more detailed analysis of cellular immune responses, animals were immunized SC in the hind footpads with 20-50 μg peptide in CFA, and draining popliteal and inguinal lymph nodes (LN) were harvested 9 days later. LN cells were restimulated *in vitro* with synthetic peptide or 25-50 μg/ml purified protein derivative (PPD, Connaught, Swiftwater, PA) in complete RPMI with 0.5% heat-inactivated autologous mouse serum (Jackson Immunochemicals, West Grove PA). On day 3, cultures were pulsed with 1 μCi/well of [^{3}H]thymidine and incubated an additional 14-20 hours, for the determination of proliferative responses. Cells were then harvested on glass fiber filters and incorporated ^{3}H was detected using a direct beta counter (Packard, Matrix 9600). IL-2 and IL-4 cytokine production was quantitated using CTLL and CT.4S bioassays, respectively, testing serial dilutions of culture supernatants. Recombinant IL-2 (Genzyme, Cambridge, MA) and IL-4 (from Dr. William Paul, NIH) were used to generate standard curves. Dilutions of anti-IL-2 mAb S4B6 and anti-IL-4 mAb 11B11 (ATCC, Rockville, MD.) were included in the assays to establish cytokine specificity.

For determination of B-cell tolerance induction, Tg mice or adoptively transferred recipients were immunized with a chemical conjugate of cysteine-modified 12-26 and HEL (12-26-HEL). Tg or NTg control mice were immunized ip with 50 μg 12-26-HEL emulsified 1:1 in CFA and then boosted with 10 μg of the same conjugate in saline 2 weeks later. Titers of IgG antibodies specific for the peptide- or HEL were determined by ELISA 8 days following this boost. Irradiated (400 rad) BALB/c recipients were adoptively transferred (iv) with 5 x 10^7 splenocytes from previously

tolerized BALB/c, and boosted ip with 100 μg 12-26-HEL conjugate in incomplete Freund's adjuvant (IFA). Serum bleeds were collected 8 days following this boost, and antibody titers determined by ELISA.

Antigen-Presentation Studies. The ability of Tg B cells to directly present endogenous 12-26 peptide was assessed with T-cell hybridoma 9C127 which recognizes 12-26 peptide in the context of l A^d. Tg or control littermate B cell APC were purified as described above, and recultured in varying numbers in 200 μl microcultures with 10^4 9C127 cells/well in complete RPMI with 5% FCS. Supernatants were harvested 48 hours later, and multiple dilutions were assayed for IL-2 production as above.

Immunologic Methods. ELISA determinations of serum peptide-specific or HEL-specific IgG responses were performed by coating plates with 50 μg/ml synthetic peptide or 5 μg/ml HEL and following standard ELISA protocols. Briefly, antigen-coated plates were blocked with 1% gelatin/0.05% Tween 20 buffer, and duplicate serial dilutions of serum were incubated and probed with goat anti-mouse IgG isotype-specific secondary reagents conjugated to alkaline phosphatase. Titers are expressed as the geometric mean of the reciprocal dilution required to bring A_{490} readings to prebleed levels or <0.09 O.D.

12-26-IgG H chain protein was detected in serum of Tg mice via its ability to bind to the NIP (5-iodo-4-hydroxy-3-nitrophenylacetyl) hapten using a modified NIP-binding ELISA (Grosschedl, R. *et al.* (1984) *Cell. 38*:647-658). Dilutions of sera from Tg mice were incubated on ELISA plates coated with NIP-gelatin or NIP-BSA conjugates (10 μg/ml), and subsequently probed with goat anti-mouse IgG_1-AP as a secondary reagent. Detection of the 12-26 epitope in Tg sera could be demonstrated by similarly using NlP-sepharose beads (from Dr. T. Imanishi-Kari, Tufts University) to immunoprecipitate 12-26-IgG. Samples were boiled in 2X SDS loading buffer, electrophoresed on 10% SDS-PAGE, and transferred onto nitrocellulose filter in a buffer with 25 mM Tris, 192 mM glycine, and 20% methanol, pH 9.0 at 20°C overnight. Blots were blocked in 2% BSA in TBST (50 mM Tris, 200 mM NaCl, pH

7.5, 0.05% Tween 20), and probed with biotinylated mAb B3.11 (anti-12-26 epitope) plus streptavidin-AP as a secondary reagent.

FACS analysis. Cells were stained for surface antigens and analyzed by flow cytometry using standard methods. The following conjugated antibodies were from commercial sources and used with appropriate fluorochrome-labeled isotype/species matched or secondary reagent controls: RA3-6B2, rat anti-mouse Ly5 (B220)-PE (Caltag, San Francisco, CA), YTS 191.1, rat anti-mouse L3/T4 (CD4)-PE (Caltag), YTS 169.4, rat anti-mouse Ly-2 (CD8)-FITC (Caltag), goat anti-mouse IgG1 (adsorbed)-PE (Caltag), goat anti-mouse IgM (H+L)- FITC (Hyclone). Data was acquired on a Becton Dickinson FACScan and analyzed with LYSIS II software.

II. RESULTS

Generation of Transgenic Mouse Lines Expressing a Novel Peptide-IgG_1 Construct Specifically in the B-Lymphocyte Compartment

The unique tolerogenic properties of an engineered peptide-IgG fusion protein expressing residues 12-26 of λ cl repressor protein at the N-terminus of a murine H chain specific for the NP hapten is described above. The engrafted epitope is recognized on the exterior surface of assembled IgG by a peptide-specific mAb (B3.11). More importantly, soluble fusion protein administered in adjuvant can efficiently generate peptide-specific T-cell responses *in vivo*, suggesting that 12-26 (or an extremely similar peptide) is processed and presented by endogenous APC, even in the context of an Ig scaffold. To further characterize the potential for expressing tolerogenic IgG fusion proteins *in vivo*, we generated Tg mice expressing the engineered genomic (rearranged) H chain construct driven by its endogenous immunoglobulin promoter/enhancer sequences (Example I). Tg founders possessing 2-3 integrated copies were identified via Southern blotting of genomic tail biopsy DNA using a cDNA probe containing 12-26 sequence (Figure 8). Two lines (5 and 17) were bred onto the BALB/c ($H\text{-}2^d$) strain and further analyzed for expression of engineered IgG. Unlike Tg mice expressing rearranged IgM constructs, both lines had no apparent suppression of endogenous Ig rearrangements and expressed amounts of surface IgM, serum levels of IgM and IgG, as well as B220, CD4, and CD8 markers

that were comparable to NTg littermates. These results are in agreement with previous observations for IgG H chain Tg experiments (Storb, U. (1987) *Ann. Rev. Immunol. 5*:151 -174; Yamamura, K. *et al.* (1986) *Proc. Natl. Acad. Sci. USA. 83*:2152-2156.; Tsao, B.P. *et al.* (1992) *J. Immunol. 149*:350-358; Radic, M.Z. *et al.* (1995) *J. Immunol. 155*:3213-3222).

Serum expression of the NP-binding Tg H chain was detected as described by Grosschedl *et al., supra.* Since the Tg V_H region binds with high affinity to the NIP hapten in combination with λ1 light chains, functional 12-26-IgG was detected indirectly with a NIP-binding IgG_1 ELISA Although probably representing a fraction (only λ light chain-associated) of expressed Tg serum protein, NIP-binding IgG_1 assays revealed that Line 17 and 5 expressed between 1000-25000 ng/ml and 50-1000 ng/ml, respectively. The higher serum expression for Line 17 mice correlated with increased expression of surface IgG_1 in splenocytes as compared to Line 5 or NTg littermates.

Direct presentation of endogenously synthesized 12-26 by Tg B cells was demonstrated with a peptide-specific hybridoma (9C127). More importantly, 12-26 peptide expression was demonstrated directly via immunoprecipitation of Tg serum with NIP-sepharose beads and immunoblot analysis with mAb B3.11. B-lymphoid expression of the transgene mediated by a specific Ig promoter/enhancer was demonstrated as significantly increased expression following activation by bacterial LPS, but not by Con A stimulation of splenocytes. These results collectively show that the Tg 12-26-IgG_1 H chain readily combines with endogenously-synthesized light chains to be expressed specifically as a self molecule by a large fraction of B cells without perturbing expression of endogenous IgM rearrangements. Thus, the 12-26 peptide is secreted as well as processed and presented via the Ig endocytic pathway as an endogenous B cell self-epitope.

Profound Peptide-Specific Cellular and Humoral Immune Tolerance in Mice Expressing 12-26-IgG_1 During Development or in Adult Bone Marrow Chimeras

Tg mice expressing foreign "neo" self-antigens have firmly established that tolerance induction can readily occur for membrane-bound and soluble proteins which

are expressed ubiquitously, or in a tissue-specific manner during normal development (Goodnow, C.C. (1992) *Ann. Rev. Immunol. 10*:489-518; Miller, J.F.A.P. *et al.* (1992) *Ann. Rev. Immunol. 10*:51-69). In our initial experiments, we wished to establish that Tg animals expressing a model immunodominant epitope as part of serum IgG protein were similarly unresponsive to an immunogenic challenge with the epitope. Since the 12-26 peptide contains both a T-cell and a B-cell epitope, we could measure both cellular and humoral immune responses to this relatively simple determinant with immunization assays. Draining LN cells from subjects who received SC injection of synthetic peptide in CFA and which were subsequently restimulated with antigen displayed a profound proliferative unresponsiveness and IL-2 production. Furthermore, NTg mice (H-2^d) primed with peptide in adjuvant and followed by a subsequent boost of peptide in saline 2 weeks later, developed an extremely high titer serum antibody response dominated by antibodies of the (Th2-mediated) IgG_1 isotype (Soloway. *et al.*, *supra*). Profound humoral unresponsiveness was observed in Tg animals immunized in this manner (Fig. 9A). This could not be due to immune-complex binding with circulating serum fIg (12-26-IgG) since these tolerant animals had diminished splenic memory T cell responses to 12-26 peptide (Figure 9B). The extent of cellular and humoral unresponsiveness was comparable for both Line 5 and Line 17 suggesting that even lower levels of expression (Line 5) efficiently satisfied antigenic thresholds for tolerance induction.

In the immunocompetent adult, solid induction of tolerance to foreign transplantation antigens or viral CTL epitopes has previously been shown to be most effective in subjects in whom hematopoietic or lymphoid ablation is followed by reconstitution with antigen-expressing BM-derived APC (IIdstad, S. T. *et al.* (1984) *Nature 307*:168-170; Cobbold, S.P. *et al.* (1984) *Nature 312*:548-551; Roberts, J.L. *et al.* (1990) *J. Exp. Med. 171*:935-940; Oehen, S.V. *et al.* (1994) *Cellular Immunol. 158*:342-352; Nemazee, D. *et al.* (1989) *Proc. Natl. Acad. Sci. USA. 86*:8039-8043). To ascertain whether tolerance to a class II-restricted T cell and B cell epitope could similarly be achieved, we constructed Tg BM chimeras.

Sublethally irradiated adult BALB/c mice were injected with 1:1 mixtures of Line 17 Tg and NTg littermate BM, and the recipient's immune system was allowed to redevelop for 7-8 weeks in the presence of fIg (12-26-IgG)-producing lymphoid tissue. Such treatment followed by immunogenic challenge with synthetic peptide revealed profoundly suppressed cellular and humoral (Figure 10) peptide-specific immunity in these normal adult recipients.

The magnitude of tolerance as well as serum NIP-binding IgG_1 levels observed for these BM chimeras was comparable to that observed in Tg animals expressing 12-26 IgG continuously during ontogeny. More interestingly, even injection of *nonirradiated* subjects with large numbers of crude syngeneic Line 17 BM resulted in high levels of serum NIP-binding IgG_1 which could be detected as long as one year post-infusion.

Tolerogenicity of fIg-Expressing Lymphoid Tissue Transferred to Unmanipulated Adult Subjects

Although tolerance in the neonate or hematopoietically-ablated adult recipient is believed to involve thymic participation (central tolerance), we analyzed the potency of peptide-fIg-expressing lymphoid tissue for the induction of tolerance in mature peripheral lymphocytes. To induce peripheral tolerance in an unmanipulated immune-competent adult, we injected iv various preparations of Line 17 Tg hematopoietic tissue into normal adult BALB/c subjects. We first compared the *in vivo* tolerogenic efficacy of injecting large numbers of resting *vs.* activated B cells. Resting B cells are known to be competent in antigen processing and presentation functions, but have been described to possess defective costimulatory ability, in contrast to LPS- or surface Ig-activated B cell blasts which express abundant B7-1, B7-2, and CD40. Surprisingly, injection of a variety of different 12-26-IgG-expressing lymphoid preparations, including Percoll gradient-purified resting B cells, LPS-activated blasts, crude BM , and even crude splenocyte preparations, were all highly effective in diminishing humoral (Figure 11) and cellular immune responses to the 12-26 peptide in adult recipients.

Analysis of B-Cell Tolerance Induction in Transgenic or Normal Tolerized Recipients

Although processed 12-26 peptide induced efficient tolerance in T cells, the bivalent epitope-containing IgG molecule could potentially induce B cell tolerance (Chiller, J.M. *et al.* (1970) *Proc. Natl. Acad. Sci. USA. 65*:551-556; Parks, D.E. *et al.* (1980) *J. Immunol. 124*:1230-1236; Tighe, H. *et al.* (1995) *J. Exp. Med. 181*:599-606). To test for the potency of such an effect, line 5 and 17 Tg mice were challenged with peptide conjugated to a different carrier, hen egg lysozyme (HEL), as a source of T cell help for potentially tolerized B cells. Potentially self-reactive anti-peptide B cells can receive foreign-reactive T cell help from HEL-specific T cells to produce autoantibodies. Immunization with 12-26-HEL in adjuvant, followed by a boost, revealed that both Tg lines displayed B-cell tolerance, manifest as a reduction in anti-peptide IgG titers (Figure 12A). In contrast, all animals expressed similar levels of anti-HEL IgG titters ($>10^5$). Interestingly, although the lower expressing Line 5 was solidly unresponsive to 12-26 when challenged with peptide alone (Figure 12B), immunization with the conjugate revealed low but significant anti-peptide responses not evident in the higher expressing Line 17. Thus, although both lines are solidly tolerant at the T cell level, a more potent B cell tolerance appeared to have been associated with higher self-peptide concentrations in Line 17 mice.

We also assessed the effect on B-cell tolerance of transferring 12-26-IgG-expressing lymphoid tissue to normal, unconditioned recipients. Normal immunocompetent subjects that were rendered tolerant by various preparations of Line 17 lymphoid cells, displayed similar levels of humoral immune tolerance (Figure 11). Since BM cells, resting B cells, and activated LPS B cell blasts secrete or express on their surface varying amounts of the fIg, it is conceivable that, although they are all effective in inducing T-cell tolerance (via similar tolerogen presentation pathways), they may have differing effects on B cell tolerance induction. Thus, we adoptively transferred splenocytes from previously-tolerized animals immunized with 12-26 peptide and HEL (as a specificity control), and which had previously displayed unresponsiveness (Figure 11), into irradiated BALB/c recipients. These recipients were boosted with peptide-HEL (to stimulate T-cell help for potentially tolerized B

cells). Anti-peptide IgG titers were measured. Surprisingly, although all animals displayed relatively lower titers than non-tolerant controls, recipients of splenocytes from LPS-blast tolerized donors appeared to have a more profound B cell tolerance (Figure 12B).

Splenocytes from tolerized animals were also restimulated *in vitro* with peptide and analyzed for IL-2/IL-4 cytokine responses. This experiment confirmed that the various lymphoid treatments resulted in similarly decreased cytokine responses , and thus similar T-cell tolerance induction as was suggested by results described above.

Induction of Tolerance in Previously Primed Adult Subjects

Various experimental models of tolerance induction have established that it is possible to diminish specific immunity in a naive, antigen-inexperienced recipient (Eynon, E.E. *et al.* (1991) *Transplant. Proc. 23*:729-730; Eynon, E.E. *et al.* (1992) *J. Exp. Med. 175*:131-138; Fuchs, E.J. *et al.* (1992). *Science. 258*:1156-1159). In contrast, inducing unresponsiveness in an antigen-primed (immunized) adult has been more difficult (Fuchs *et al., supra;* Eynon, E.E. *et al.* (1993) *J. Immunol. 151*:2958-2964).

We tested the ability of tolerogen-synthesizing B cells to modulate an ongoing immune response by SC immunization of recipients with peptide in adjuvant and waited 1-2 weeks *before* injecting of Line 17 Tg B cell preparations as tolerogen. Primed recipients received one of four preparations: (1) Percoll purified resting B cells, (2) crude BM cells, (3) LPS-activated B cell blasts, or (4) chemically-fixed B cells. One week later, they were boosted with peptide in saline, and humoral immune responses were subsequently determined.

Although both resting B cells and crude BM cells produce specific unresponsiveness in antigen-naive recipients, both were ineffective in diminishing peptide-specific humoral immunity in previously primed subjects (Figure 13A). LPS-activated Tg B cells completely reversed the ongoing immune response (Figure 16B). A significant reduction in anti-peptide antibody titers was also produced by treatment with fixed Tg B cells (Figure 13C). Thus, the most potent tolerogenic treatment for

already immune subjects, which caused them to become as unresponsive as antigen-naive subjects, was the infusion of activated fIg-synthesizing B cells. Furthermore, diminution of anti-peptide antibody responses by activated or fixed fIg-expressing Line 17 B cells was observed not only for total IgG levels. Rather antibodies of the IgG_1 isotype, a Th1-dependent response, and antibodies of the IgG_{2b} isotype, a Th2 dependent response, were also diminished, thus ruling out a possible "immune-deviation" or class-switching effect (Asherson, G.L. *et al.* (1965) *Immunology. 9*:206-215).

III. DISCUSSION

A variety of protein engineering strategies have established the efficacy of expressing heterologous epitopes in immunoglobulin frameworks for the enhancement of specific immunity (Billetta, R. *et al.* (1991) *Proc. Natl. Acad. Sci. USA. 88*:4713-4717.; Zaghouani, H. *et al.* (1993) *Science. 259*:224-227). The present inventors have shown (see Example I) that similarly constructed peptide-Ig molecules can induce specific tolerance to a foreign immunodominant epitope. The above studies extend this approach to a novel strategy in which antigen-presenting B cell may be engineered to express immunoglobulins which contain within their structure tolerogens that can be employed to manipulate an undesired immune responses. Expression of otherwise immunogenic determinants in an IgG fusion protein which is synthesized, secreted, and also directly presented by lymphoid tissue, was shown to be a highly efficient tool for the induction of immune self-tolerance in mature, immunocompetent subjects.

In the model described herein , iv injection of any of a variety of crude or purified lymphoid cell preparations were effective for tolerance induction. We were surprised to find that even unfractionated splenocytes were effective, since such a population includes dendritic cells which have been reported to prevent induction of unresponsiveness (to the H-Y antigen) (Fuchs *et al.* supra). This is most likely due to the restricted expression of the self-antigen in the present system to the B-lymphoid cell compartment whereas H-Y is expressed by B cells and other APC.

Transgenic peptide-Ig chimeric molecules have the potential to be presented directly or secreted and re-presented, making it likely that tolerance induction by injected of peptide-expressing lymphoid tissue occurs via multiple pathways. This may also explain the potency of the fIg tolerogens. Secretion of the fIg tolerogen by activated transgenic B cells and re-presentation by non-transgenic APC may provide an additional tolerogenic pathway. This is supported by our observations that high doses of soluble peptide-IgG, or very low doses of fIg from secreting transfected cells, upon injection d *in vivo*, are sufficient for inducing tolerance (Zambidis *et al., supra*). T-cell clonal deletion has been described in other transgenic models in which soluble self-Ig antigenic determinants were presented in the periphery or in the thymus (53, 64). Thus, although direct presentation of self-antigens by B cells may be sufficient for peripheral tolerance induction, additional pathways using other APC such as macrophages (especially for soluble IgG antigens) (Phillipis, J.A. *et al.* (1996) *J. Exp. Med. 183*:1339-1344) may also tolerize independently.

BM-derived B cells or purified resting B cells are deficient in costimulatory function. Hence, for these cells, direct presentation of endogenously synthesized 12-26 peptide (signal 1) is the most likely primary tolerogenic pathway. Such cells express very little membrane or secreted IgG_1. Thus, a relatively low level of production of soluble and/or membrane fIg capable of interacting with surface IgM molecules specific for the foreign epitope may explain why such cells were relatively less tolerogenic for the B cell compartment. In contrast, activated B cells with increased secretion of fIg were more efficient B cell tolerogens, and were the only preparation we tested which could shut down an ongoing immune response.

Recently, high doses of antigen given to naive or even primed recipients was shown to cause clonal deletion of both peripheral T and B lymphocytes via mechanisms of programmed cell death, or apoptosis. Peripheral deletion of mature lymphocytes resulted from an exhaustive immune response ("propriocidal regulation") which was IL-2-dependent and mediated by the apoptosis-regulating surface molecules Fas and Fas ligand (Crispe, I.N. (1994) *Immunity. 1*:347-349; Critchfield, J.M. *et al.* (1994) *Science. 263*:1139-1143; Singer, G.G. *et al.* (1994)

Immunity. 1:365-371; Pulendran, B. *et al.* (1995) *Nature 375*:331-334; Shokat, K.M. *et al.* (1995) *Nature 375*:334-338; Lenardo, M.J. (1991) *Nature 353*:858-861).

One interpretation of the present findings is that prolonged presentation of peptide/MHC complexes provided by live transgenic B cells, combined with sustained production and re-presentation of secreted flg activates those mechanisms responsible for high dose tolerance. The existence of an activation-driven apoptosis mechanism may explain our observation of tolerance induction in subjects during an ongoing immune response when treated with transgenic LPS blasts expressing the flg.

Abundant evidence exists that transmittal of signal 1 alone (for example by using fixed APC) may be important in tolerance induction *in vitro* or *in vivo* of preactivated T cell clones, or tolerance induction in a naive immune system. We now add the notion that resting or fixed (costimulation-deficient) transgenic B cells, which are not expected to secrete significant amounts of Ig, may evoke a different tolerogenic pathway than does a large dose of activated B cell APC injected iv. In the present studies, resting B cells were less efficient at curtailing an ongoing immune response.

It is concluded that the expression of a flg construct comprising a selected foreign epitope or epitopes in peripheral B cells using gene therapy strategies has great practical utility for modulating humoral and cellular immune responses. In comparison to currently used methods of high dose tolerance or oral tolerance, genetic transfer and expression of tolerogens in lymphoid APC requires only knowledge of the DNA sequence encoding the target epitope towards which tolerance is desired. The present method avoids the cumbersome antigen purification/synthesis steps . More importantly, since clinically useful tolerance would require that the antigen (tolerogen) persist, its genetic expression in long-lived APC or pluripotential hematopoietic stem cell precursors provides a means for achieving the requisite persistence. The present inventors have also induced peptide-specific tolerance by expression a flg construct in peripheral B cells or hematopoietic stem cells using retroviral-mediated gene transfer.

EXAMPLE IV

Genetically-Transferred Central and Peripheral Immune Tolerance Via Retroviral-Mediated Expression of Immunogenic Epitopes in Hematopoietic Progenitors or Peripheral B Lymphocytes

One potential strategy for the induction of clinically relevant tolerance is indirectly related to the original demonstration by Medawar's group of tolerance induction to foreign MHC antigens via injection of allogeneic hematopoietic cells into neonates (Billingham *et al., supra*). In adults, attempts to induce tolerance to foreign grafts by injecting accessory-cell depleted splenocytes (Ryan, J.J., *et al.* (1984) *J. Immunology 133*:2343-2350; Hori, S., *et al.* (1989) *J. Immunology 143*:1447-1452) or syngeneic transfected cells (Madsen, J.C., *et al.* (1988) *Nature 332*:161-164) has met with, at best, limited success. The advent of efficient methods for gene transfer into hematopoietic cells may, in theory, enable the expression of foreign antigens for the induction of tolerance via pathways similarly used for tolerance induction to naturally-expressed epitopes (*e.g.* MHC, Mls antigens) in bone marrow chimeras (Ramsdell, F., *et al.* (1989) *Science 246*:1038-1041; Roberts, J.L., *et al.* (1990) *J. Exp. Med. 332*:161-164; Gao, E-K, *et al.* (1990) *J. Exp. Med. 171*:1101-1121; Sachs, D.H., *et al.* (1993) *Transplantation Proc. 25*:348-349). Since such approaches have usually required some degree of myeloablation, a more desirable approach would be to adoptively transfer genetically modified peripheral APC (Sutkowski, N., *et al.* (1994) *Proc. Natl. Acad. Sci. USA 91*:8875-8879), which engraft more efficiently in an unconditioned host. An excellent candidate for such a strategy would be the peripheral B lymphocyte which has been described to possess immune-modulating characteristics. Previous studies have shown that antigen-presenting B cells are capable in some circumstances of inducing peripheral tolerance of (a) mature, naive T cells *in vivo* (Webb, S., *et al.* (1990) *Cell 63*:1249-1256; Eynon, E.E., *et al.* (1992) *J. Exp. Med. 175*:131-138; Fuchs, E.J., *et al.* (1992) *Science 258*:1156-1159; Buhlmann, J.E., *et al.* (1995) *Immunity 2*:645-653), or (b) previously activated T cell clones *in vitro* (Gilbert, K.M., *et al.* (1994) *J. Exp. Med. 179*:249-258).

To test the potential for gene-transfer of a target antigen into autologous APC for the induction of specific immune tolerance, we created a recombinant, replication-

defective retroviral vector for the expression of a foreign class II MHC-restricted immunodominant model epitope, 12-26 (Soloway, P., *et al.* (1991) *J. Exp. Med. 174*:847-858; Lai, M-Z, *et al.* (1987) *J. Immunology 139*:3973-3980), fused at the N-terminus of a murine IgG H chain, as described above. Engineered Ig expressing heterologous epitopes has been described for the potentiation of peptide-specific immunity (Zaghouani, H., *et al.* (1993) *Science 259*:224-227), and the Examples above expanded this approach by describing the tolerogenic properties of a soluble engineered 12-26-IgG fusion protein. The genetic transfer and expression of immunogenic epitopes, or whole complex antigens by appropriate "non-professional" APC has great utility for the specific elimination of undesirable immunity associated with HIV infection, as describe herein, autoimmune states (Tisch, R. *et al. supra*; Higgins, P.J. *et al. supra*; Critchfield, J.M., *et al., supra*), recombinant clotting factor administration (Allain, J.P. *et al.* (1976) *Blood 47*:973), and gene therapy protocols (Yang, Y., *et al.* (1995) *J. Virol. 69*:2004-2015; Tripathy, S.K. *et al.* (1996) *Nature Medicine 2*:545-550).

A. Materials and Methods

1. Replication-defective retroviral vectors and gene-transfer protocols

12-26-IgG_1 H chain cDNA was derived by RT-PCR from J558L myeloma cells, transfected with the rearranged genomic construct (Examples I-III; Zambidis *et al., supra*) and subcloned into retroviral vector MBAE (Kang, J., *et al.* (1990) *Proc. Natl. Acad. Sci. USA 87*:9803-9087) containing long terminal repeats (LTR), ψ+ packaging signals, a neomycin resistance gene, and cloned human b-actin promoter sequences. PCR primers encoded 5' Ig H chain leader and 3' IgG_1 as well as Sal I restriction site sequences:

V_H 5' primer: TGGACTAAGTCGACACCATGGGATGGAGC (SEQ ID NO:207)
G1 3' primer: TCGGAAGGGTCGACGGATCATTTACCAGGAGA (SEQ ID NO:208)

A high titer (10^5-10^6 neomycin-resistant NIH 3T3 CFU/ml) ψ-2 packaging line (F6P) was prepared with recombinant plasmid MBAE.BAK, and assayed for helper virus via horizontal spread of neomycin resistance with NIH 3T3 cells. Ecotropic F6P was prepared by "ping-pong" amplification using amphotropic line PA317. Producer lines

were stored in liquid nitrogen and freshly thawed cells were utilized for each individual experiment.

B cell lines CH31, A20, J558L, and NS-1 (ATCC, Rockville, MD) were transduced with recombinant retrovirus via co-culture with adherent F6P cells for 24-48 hours in the presence of 6 μg/ml polybrene (Sigma). Cells in suspension were washed and recultured in 1 mg/ml G418 for selection of stable transductants prior to genomic Southern blot, RT-PCR, ELISA, or antigen-presentation studies. Infection of BM progenitors and quantitation of G418-resistant colony-forming cells (CFC) has been described (Keller, G., *et al.* (1985) *Nature* 318:149-154; Bodine, D.M., *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:8897-8901). BM was harvested from femurs and tibiae of 6-8 week old BALB/c donors injected IV with 150 mg/kg 5-fluorouracil 3-4 days previously. Erythrocyte-depleted BM was co-cultured ($5x10^6$/ml) with irradiated (2000 rads) F6P or ψ-2 parental cells (mock transduction). Ten ml cultures in complete RPMI 1640 with 15% FCS were incubated at 37° C, 5% CO_2 for 48 hours, and included 200 U/ml each of IL-3, IL-6, and IL-7 (Genzyme). 4 μg/ml polybrene was added to co-culture during the last 24 hours of infection.

Splenic B cells were similarly infected *in vitro* via co-culture with viral-producing F6P or parental ψ-2 (mock transduction). Peripheral B cells were purified with anti-T cell antibody cocktail plus complement and Percoll density gradients (60-70% layers). Purified B cells were pre-stimulated with 50 μg/ml bacterial lipopolysaccharide (LPS, E. coli 055:B5, Sigma) overnight, and recultured ($3x10^6$/ml, 5 ml cultures) with irradiated F6P in the presence of 4 μg/ml polybrene and 50 μg/ml LPS for an additional 24 hours.

2. Tolerance induction and measurement of peptide-specific cellular and humoral immunity

Adult (6-8 week old) BALB/c recipients (Jackson Labs) were sublethally irradiated (200-600 rads total body irradiation) and injected intravenously (lateral tail vein) with $1-2x10^6$ gene-transferred or mock-transduced BM progenitor cells. Unconditioned, normal BALB/c were similarly injected with $>1x10^7$ gene-transferred LPS blasts. All cells were washed extensively in serum-free medium and injected IV

in a volume of 500 µl. Recipients were analyzed for expression of recombinant retrovirus as well as immune tolerance to 12-26 peptide 2-12 weeks later. Tolerized recipients were immunized for cellular or humoral immune responses as described above (see also: Soloway, P. *et al.* (1991) *J. Exp. Med. 174*:847-858; Zambidis *et al.*, supra). Animals were injected SC with 50 µg synthetic 12-26 peptide emulsified 1:1 CFA. and in some experiments, also ip with 10 µg hen egg lysozyme (HEL) in CFA as a specificity control. Two weeks later, mice received an additional ip boost of 50 µg peptide and 10 µg HEL in saline. Antibody titers were determined from serum bleeds 8 days after secondary boosts. Splenic memory T cell responses were measured *in vitro* 6-8 weeks following these secondary challenges by reculturing purified T cells ($3x10^6$/ml) with irradiated (2500 rads) BALB/c splenocytes ($1x10^6$/ml) and dilutions of synthetic peptide. Serum peptide-specific or HEL-specific IgG responses were determined by ELISA as described (supra). Cellular responses from draining popliteal and inguinal LN cells were assayed 9 days after SC immunization with 20 µg peptide in CFA. Cultures were pulsed with [^{3}H]thymidine, harvested and counted as described above) IL-2 and IL-4 cytokine production was quantitated as above. Dilutions of anti-IL-2 mAb S4B6 and anti-IL-4 mAb 11B11 (ATCC) were included to confirm specificity. IFN-γ was measured using a commercial ELISA kit (Intertest-γ, Genzyme).

3. RT-PCR and immunologic methods

Detection of 12-26-IgG transcripts in transduced cell lines or hematopoietic tissue from BM-injected mice was performed with a 12-26 sequence RNA-PCR assay. Primers were designed to amplify 5' immunoglobulin leader sequence, "V_H 5' primer", as above, and 3' 12-26 sequence, ("3' pep primer"):

GGC AAC AGA AGC TTT CAC TTC TTC TTC TCG TAT (SEQ ID NO:209).

Briefly, 1-5 µg of total RNA from various tissue was reverse-transcribed (2 rounds) with AMV reverse transcriptase, dNTP's, and oligo dT and random hexamer primers (Invitrogen cDNA cycle kit) at 42° C. The resultant cDNA was amplified with 5' and 3' primers described above and Taq polymerase (Perkin-Elmer Cetus). PCR

conditions were 45 seconds at 93° C, 2 min at 47° C, and 2 min at 72° C for 35-40 cycles. Amplified DNA products were loaded (1/10-1/100 sample) onto 2% agarose/TBE gels and subsequently transferred onto nylon membranes for Southern blot analysis. 12-26 sequences were confirmed in RT-PCR amplified DNA samples with a g-^{32}P-labeled oligonucleotide ("oligo Ig-one") encoding 12-26 but which does not overlap with the 3' PCR primer: TGATCTACTGCAGCTGGAGGACGCGCGGCGG (SEQ ID NO:210). Tissue RNA samples were compared via b-actin RT-PCR using commercially available primers (Stratagene).

12-26-IgG H chain protein was detected in culture supernatants of transduced cell lines, or in sera of mice injected with gene-transferred cells, via its ability to bind to the NIP hapten using a modified NIP-binding ELISA as above. Briefly, dilutions of culture supernatants or sera were incubated on ELISA plates coated with NIP-gelatin conjugate (and subsequently probed with goat anti-mouse IgG_1-AP. Standard curves with affinity-purified 12-26-IgG from supernatants of transfected J558L were used for quantitation.

B. Results and Discussion

A recombinant retroviral vector (Kang, J., *et al., supra*) was modified by inserting a PCR-derived cDNA encoding the 12-26-IgG H chain sequence (Figure 14), and a high titer ecotropic packaging line (F6P) was generated for the *in vitro* infection of cell lines and hematopoietic tissue via co-culture methods (Keller, G., *et al.* (1985) *Nature 318*:149-154; Dick, J.E., *et al.* (1985) *Cell 42*:71-79; Bodine, D.M., *et al.* (1989) *Proc. Natl. Acad. Sci. USA 86*:8897-8901). For initial studies, a variety of B cell lines at various stages of differentiation were transduced, including CH31 (immature), A20 (mature, activated), NS-1, and J558L (plasmacytomas). Intact proviral integration in transduced G418-resistant A20 cells could be verified by genomic Southern blotting using a DNA probe specific for 12-26 sequence (Figure 14). More importantly, 12-26-IgG H chain can assemble with endogenous light chains in transduced B cell lines, to be expressed as a membrane surface protein, or secreted into cultured supernatants (50-80 ng/ml) in NS-1 and J558L myelomas. Immunoprecipitation of secreted 12-26-IgG and immunoblot analysis with a peptide-

specific monoclonal antibody (B3.11) could directly demonstrate the expression of 12-26 peptide.

Although retrovirally-synthesized gene products are expected to give rise primarily to processed peptides presented by MHC class I molecules, endogenously-derived peptides can also be routed to endocytic class II MHC compartments in some cases (Weiss, S., *et al.* (1991) *Cell 64*:767-776). Such a pathway should be enhanced for retrovirally-encoded 12-26-IgG H chain due to the efficient nature of the Ig secretory pathway in targeting the endosomal compartment. To test for direct presentation of this model immunodominant class II-restricted peptide, we tested the ability of 12-26-IgG-transduced A20 cells to directly activate peptide-specific T-cell hybrid 9C127, which recognizes 12-26 in the context of I-A^d (Lai, M-Z, *et al., supra*). Efficient presentation of endogenously synthesized peptide was demonstrated, and this effect was blocked with antibodies to CD4 or class II MHC molecules. These results predict that, *in vivo*, the 12-26 peptide could be recognized directly by T cells from a variety of gene-transferred APC (both lymphoid and non-lymphoid). Additionally, synthesis, L chain assembly and secretion of 12-26-IgG H chain by B cells can potentially result in re-presentation of the molecules by endogenous host APC.

The next experiments tested the potential of genetically modified BM cells to specifically tolerize a regenerating immune repertoire ("central tolerance" induction). BM chimeras were produced in sublethally irradiated (200-650 rads) BALB/c mice by infusing 5-fluorouracil (FU)-pretreated donor BM which had been co-cultured with F6P. This protocol leads to newly developing lymphocytes and APC (lymphoid and non-lymphoid) that are derived from both the host, as well as the transplanted BM progenitors expressing 12-26-IgG. Mice were immunized 4-12 weeks post-infusion and specific immune responses were measured. Analysis of hematopoietic tissue (collected at sacrifice) using a peptide sequence-specific RT-PCR assay indicated that transcripts were expressed consistently and reproducibly in the BM of all gene-transferred recipients (Figure 15), although variably in the thymus or spleen. A very sensitive NIP-binding IgG_1 ELISA was used that detects secreted 12-26-IgG in the serum which is a result of pairing of the fusion protein H chain with endogenous λ L

chains in B cells derived from the gene-transferred stem cells. Although this assay detects only a fraction of H chain secreted by B cells (λ light chain-paired), expression could be detected in approximately one-third of all gene-transferred BM recipients (Table V). Despite these variable serum expression patterns, dramatic and reproducible peptide-specific tolerance was observed in all 22 studied recipients of 12-26-expressing progenitor cells, which tolerance was demonstrated following immunization with synthetic peptide in adjuvant. Specific T-cell proliferative and cytokine responses of draining LN cells were significantly diminished (Figure 16), as were anti-peptide antibody levels following priming and boosting (Figure 17A,B). Since the present BM transduction protocol was designed to effect viral integration into early hematopoietic progenitors, and both the 5' viral LTR and β-actin promoters are non-specific as to cell lineage, a variety of differentiated cells with APC capacity may directly tolerize developing lymphocytes. We predicted that non-lymphoid APC derived from transduced stem cells (*e.g.* monocytes, macrophages, dendritic cells), which cannot synthesize Ig L chains, and thus cannot secrete the flg, may nevertheless play a critical role in direct presentation of transgenic peptide to developing lymphocytes. To test this, syngeneic BM from SCID mice was gene-transferred the tolerogenic activity of myeloid APC was analyzed. Although hematopoietic tissue from SCID mice is deficient in developing mature lymphoid cells, the APC function of cells of the myeloid (non-lymphoid) lineage remains intact (Dorshkind, K. *et al.* (1984) *J. Immunology 132*:1804-1808). 5-FU-pretreated SCID or normal BALB/c donor BM cells were co-cultured with F6P, and stem cells were injected into sublethally irradiated normal BALB/c recipients. (With SCID BM donors, normal lymphocytes can regenerate only from the recipients' stem cells). BM chimeras were rested over 2 months and subsequently immunized with 12-26 peptide for measurement of humoral immune tolerance.

Peptide-specific tolerance was comparable in recipients of either normal or lymphoid-deficient BM. In contrast recipients of mock-transduced BM (not expressing the 12-26 peptide from the flg) had high-titer antibody responses (Figure 17A,B). Analysis of G418-resistant hematopoietic colony forming cells (CFC) from

recipients of transduced normal BM or transduced SCID BM showed that the proportion of BM-derived myeloid stem cells expressing the fIg construct was similar (1-5%, Table V). These results indicate that in addition to tolerogenesis by lymphoid APC, transduced myeloid BM-derived APC can also share this activity. This result may explain the consistently solid tolerance observed in all recipients of transduced normal BM, regardless of detectable levels of 12-26-IgG (the B-lymphocyte-derived transgene) in the subjects' serum.

Although efficient induction of tolerance in newly arising lymphocytes was readily and reproducibly accomplished with genetically-modified BM, a more clinically practical approach for gene-transfer tolerogenesis would be the induction of peptide-specific peripheral tolerance in a mature immune repertoire without the need for prior myeloablation. It is known that potent tolerance in normal, immunocompetent subjects can be induced by injecting large numbers of mature B lymphocytes expressing a "foreign" antigen (*e.g.,* H-Y or Mls). Therefore, the present inventors tested the tolerogenicity of fIg -transduced peripheral B cells.

The approach comprised stimulating Percoll® gradient-purified splenic B cells to proliferate with bacterial LPS, brief co-culture with F6P, and subsequent iv injection into normal, immunocompetent (non-irradiated) BALB/c recipients. This treatment resulted in an efficient suppression of peptide-specific humoral immunity comparable to that observed in the BM chimera experiments described above.

TABLE V

Expression of Serum 12-26-IgG and G418-resistant BM Progenitors in Genetically-Tolerized Mice

Mouse #	BM Gene Transfer	Serum NIP-Binding IgG1 (ng/ml)		% $G418^R$ CFC/ml	Tolerance Induction
Expt. 1					
1, 2, 3	-	<0.1	3, 7 wks)	NT*	-
4	+	**20, 2**	(3, 7 wks)	NT	+
5	+	<0.1, **20**	(3, 7 wks)	NT	+/-
6	+	**60, 2**	(3, 7 wks)	NT	+
7	+	**120, 20**	(3, 7 wks)	NT	+
8	+	**10,** <0.1	(3, 7 wks)	NT	+
Expt. 2					
9, 10, 11	-	<0.1	(6 wks)	NT	-
12	+	**135**	(6 wks)	NT	+
13	+	<0.1	(6 wks)	NT	+
14	+	**15**	(6 wks)	NT	+
15	+	<0.1	(6 wks)	NT	+
16	+	<0.1	(6 wks)	NT	+
Expt. 3					
17, 18, 19	-	<0.1	(3 wks)	NT	-
20	+	<0.1	(3 wks)	NT	+
21	+	**30**	(3 wks)	NT	+
22	+	<0.1	(3 wks)	NT	+
Expt. 4					
23, 24, 25	-		NT	0% (11 wks)	-
26	+		NT	2.2% (11 wks)	+
27	+		NT	2.8% (11 wks)	+
28	+		NT	2.8% (11 wks)	+
29	+ **(SCID)**		NT	5.4% (11 wks)	+
30	+ **(SCID)**		NT	1.3% (11 wks)	+
31	+ **(SCID)**		NT	4.7% (11 wks)	+

Legend to Table V: Recipients of F6P or mock-infected 5-FU-treated BM progenitors were assayed for transgene expression in serum or BM at indicated times. BM CFC were assayed at sacrifice time in 0.3% semisolid agar cultures in long-term recipients. BM cells were cultured at 10^6 /well in complete IMDM plus 15% FCS, 200 U/ml IL-3, and 10% ORIGEN conditioned medium (GIBCO, BRL) containing IL-1, G-CSF, GM-CSF, M-CSF, and IL-6. Erythro-myeloid colonies were grown with and without 1 mg/ml G418, and the percentage which were viable and neomycin-resistant ($G418^R$) were counted after 7-10 days. BM recipients were conditioned with either 200 rads (Expt. 1) or 600 rads (Expts. 2-4). Detailed experimental results for Expt. 1 and 4 are presented in Figures 17A and 17B, respectively. * NT: not tested

Splenic memory T cell responses measured approximately 3 months after immunization in these subjects were markedly reduced (IL-2 and IL-4 cytokine reduction), indicative of effective tolerization in both T helper cell compartments (Th1 and Th2). See Figure 18). Furthermore, G418-resistant hybridomas could be generated from LPS-activated spleen cells of these tolerized mice by fusing the spleen cells with A20 lymphoma cells in the presence of PEG. These hybrids stimulated peptide-specific 9C127 T cells directly (Figure 19), thus proving that gene-transferred peripheral B cells are capable of persisting and presenting 12-26-IgG self-antigen for prolonged periods in a way which results in induction and maintenance of peptide-specific tolerance. Such long-term persistence (>3-6 months) is consistent with results obtained after injection of either normal peripheral B cells (Sprent, J. *et al.* (1991) *J. Exp. Med. 174*:717-728) or genetically-modified LPS-activated peripheral B cells (Sutkowski, N., *et al., supra*).

The foregoing results show that the present inventors have in hand a novel, efficient strategy for delivery of a foreign peptide, which would otherwise be an immunogen, to an adult immune system in a tolerogenic manner in the form of a soluble fIg protein expressed in hematopoietic tissue. Genetic transfer of a selected target single or multi-epitope sequence into a multipotential stem cell or into a peripheral B cell permits the induction of, and more importantly, the long-term maintenance of, specific immune self-tolerance in the autologous host. The choice of a model immunodominant peptide, 12-26, capable of inducing both high titer IgG antibody responses (Th2-mediated), as well as vigorous cellular (Th1-mediated) responses underscores the versatility of the present method. Although gene-transfer of BM expressing class I MHC-restricted CTL epitopes efficiently induced tolerance (Ally, B.A., *et al.* (1995) *J. Immunology 155*:5404-5408), the present approach of fusing an antigenic sequence to an Ig molecule allows for the efficient presentation of a retrovirally-synthesized class II MHC-restricted epitope. Furthermore, in addition to effective Th tolerance induction, the bivalent nature of the secreted form of the tolerogenic epitope on the two H chains of the Ig-molecule can independently mediate effective peptide-specific B cell tolerance, probably via Fc-mediated antibody

feedback mechanisms (Zambidis *et al., supra*). Thus, the potency of tolerance induction using the present invention can exploit multiple pathways in the immune mechanism.

The use of tolerogenic peptide-Ig constructs facilitates "tailor-designing" the immune response to a whole antigen by selectively inducing immunity (Zaghouani, H., *et al., supra*) or tolerance to selected epitopes be they immunodominant or cryptic. In contrast to expressing a heterologous epitopes in the CDR3 region of the Ig H chains, fusing an foreign antigenic sequences at the N-terminus is not limited by size restrictions, and can thus be adapted for expressing large multi-epitope antigens, for example, autoantigenic proteins such as factor VIII (Allain *et al., supra*), myelin basic protein (Higgins *et al., supra*; Critchfield *et al., supra*), or glutamic acid decarboxylase (Tisch *et al., supra*). Delivery of the tolerogen as a gene sequence has many advantages over present tolerance induction protocols, since only the cDNA sequence of the target antigen, for example, one or more HIV gp120 epitopes, needs to be known. This avoids the need for a protein purification strategy. More importantly, since experimentally acquired tolerance eventually wanes, expression and persistence of the tolerogen in long-lived or multipotential hematopoietic tissue has the potential to modulate permanently a specific immune response.

As described herein, an important application of the genetic tolerogenesis method of the present invention is to help eliminate genetically-altered cells encountered in gene therapy protocols. Autologous cells genetically modified with adenoviral and retroviral vectors are known to induce immunity in a competent recipient due to immune recognition of vector-encoded products leading to subsequent elimination of transduced cells via both cellular and humoral immunity (Yang *et al., supra*). Although, for example, immunity to low-level expression of viral proteins of first-generation, E1-deleted adenovirus can undoubtedly be reduced with further genetic manipulation of the vectors, rejection of the foreign transgenes expressed by such vectors remains an even more significant obstacle (Tripathy *et al., supra*). The present results suggest that tolerogenic pretreatment of immunocompetent recipients with vector-transduced autologous APC expressing viral or foreign transgenes will

allow the prolonged expression and multiple administration of therapeutic transgenes in immunocompetent recipients without need for generalized immunosuppressive drugs

Although solid evidence exists for the tolerogenic role of lymphohematopoietic APC in irradiated bone marrow chimeras,

The foregoing studies exploited the ability of mature peripheral B cells to induce efficient peripheral tolerance in unconditioned adults. These findings represent the first example of using transduced LPS blasts as tolerogenic vehicles. Others have reported tolerance induction via presentation of antigen by resting B cells to naive T and have implicated the poor expression of costimulatory molecules such as B7-1 and B7-2 (Hathcock, K.S., *et al.* (1994) *J. Exp. Med. 180*:631-640). Antigen-presentation by resting B cells has thus far been successful in inducting tolerance in naive recipients, but has proven ineffective in primed (Fuchs *et al., supra*) or allo-MHC-reactive recipients (Buhlmann *et al., supra*) unless an anti-gp39 (CD40-ligand) antibody was simultaneously injected to prevent upregulation of B cell costimulatory function. Paradoxically, costimulation-competent LPS blasts, as in the present experiments, could serve as efficient tolerogenic APC *in vivo* in antigen-naive recipients, or could induce tolerance *in vitro* in previously activated T cell clones (Gilbert *et al., supra*).

In studies with transgenic mice expressing the flg construct specifically in the B cell compartment, both purified resting flg-expressing B cells or their LPS-activated counterparts were highly tolerogenic in normal, antigen-naive adults. In contrast, only the activated transgenic B cells were effective in tolerizing an ongoing response in a previously-immunized recipient.

EXAMPLE V

HIV gp120 Crosslinking In Vivo Induces apoptosis of T Cells

Studies were performed in mice transgenic for the human CD4 gene. Normal BALB/c mice or mice transgenic mice for human CD4 ("CD4hu") were immunized with 20 μg of gp120 in complete Freund's adjuvant, boosted with gp120 in incomplete adjuvant and then injected intravenously with 1 μg of gp120 in PBS. Peripheral blood

lymphocytes were harvested at various times after the last injection (of soluble gp120). Total number of T cells in the peripheral blood were evaluated using flow cytometry to enumerate $CD3^+$ cells. Table VI, below shows the results as percent of total blood lymphocytes which are $CD3^+$

Table VI

HIV gp120 crosslinking induces apoptosis of CD3 cells *in vivo* in human CD4 transgenic mice

	Number of CD3+ cells in PBL					
	Days after gp120					
Donor mice	**0**	**3**	**6**	**9**	**25**	**73**
Immunized CD4hu	46	43	*23*	37	45	49
Non-immune CD4hu	44	49	53	45	49	45
Immunized BALB/c	41	43	57	48	48	47
Non-immune BALB/c	61	62	66	71	59	47

The only significant reduction in numbers of T cells was observed in the transgenic mice which had been immunized, that is, mice expressing the human CD4 molecule which can bind gp120 or gp120-anti-gp120 complexes.

Experiments were performed to study the apoptosis resulting from the ligation of T cell receptors. Mice were immunized as above. Spleens were harvested 9 days after this last injection and were cultured with medium or with anti-CD3 mAb (145.2C1; 50 μg/ml coated wells) for 24 hours; cells were then harvested, fixed and assayed for apoptosis by propidium iodide uptake and flow cytometry. The percent of hypodiploid, apoptotic cells at 24 or 48 hours with anti-CD3 and at 24 hours with anti-IgM are shown in Table VII.

Table VII

Induction of Apoptosis After T Cell Receptor Ligation

	Percent Apoptotic Cells at 24 or 48 Hours				
Donor and treatment	Bkgrnd	Medium		Anti-CD3	
	0h	24h	48h	24h	48h
gp120-immunized — CD4hu	5.9	39.0	47.0	**41.1**	***61.2***
Non-immune — CD4hu	1.7	29.5	58.0	29.0	24.6
gp120-immunized — BALB/c	0.8	38.8	42.6	18.1	16.1
Non-immune — BALB/c	2.1	51.6	47.0	24.9	8.8

"Bkgrnd" = Background values of apoptosis of freshly isolated cells (as opposed to cells cultured 24 or 48 hours).

The results show that apoptosis is increased significantly only in T cells of immunized CD4 transgenic mice whose T cells receptors have been ligated polyclonally with anti-CD3 mAb. This effect is most dramatic after 48 hours .of culture.

Collectively, the foregoing results prove that gp120 epitopes have the capacity to prime T cells for apoptosis shown as direct observation of apoptosis *in vitro* and as a loss of T cells *in vivo* which occurred selectively only in subjects who were both immunized with gp120 and who had the appropriate target T cells (bearing transgenic human CD4) to which complexes of gp120 and anti-gp120 antibodies could bind,. These results further support the findings of Finkel's group cited above and provide an even stronger basis for the utility of inducing epitope specific tolerance as described herein.

EXAMPLE VI

Ongoing Immune Responses to HIV gp120 in Human CD4 Transgenic Mice Contributes to T Cell Decline upon I.V. Administration of gp120

(most references are cited in this section as numbers and appear in a list at the end of the section)

The HIV retrovirus interacts with the host immune system in a puzzling way. Virtually everyone infected with the virus synthesizes antibodies directed against a

number of the viral envelope epitopes. However, much of this humoral response has little if any protective value over the course of HIV pathogenesis [5-16]. Titers of neutralizing antibodies in AIDS patients are low [6], and the antibodies might cross-react with self-components due to molecular mimicry and structural/genetic similarities [7-9]. Furthermore, crosslinking CD4 by anti-CD4 antibodies or gp120 and anti-gp120 antibodies can upregulate Fas expression and prime Th cells for activation-induced apoptosis [1, 10-16].

Crosslinking of human CD4 coreceptor (huCD4), by HIV gp120 and anti-gp120 antibody *in vitro* as well as *in vivo*, upregulate Fas (CD95) expression and prime T cells for activation-induced apoptosis (Banda *et al.*, *supra*; Wang *et al.*, *supra*). Based on these observations, we hypothesized that an ongoing humoral immune response to gp120 might not serve the host in a protective or virus-neutralizing manner upon exposure to HIV gp120. Rather , the response might sensitize even noninfected cells for apoptosis. Immunization mice transgenic for huCD4 ("huCD4 Tg") and control mice with 20 μg gp120 in CFA led to titers exceeding $1:10^5$ within three weeks. We injected i.v. 1 μg of $rgp120_{SF2}$ into huCD4 Tg and non-transgenic BALB/c and BALB/c x C57Bl/6 F_1 mice (CB6 F1) mice that had been immunized with $rgp120_{SF2}$. The same amount of rgp120 was also administered i.v. to unprimed huCD4 Tg mice and nontransgenic controls. Boosting gp120-primed *control* mice with gp120 gave rise to increased numbers of T and B cell as well as in the antibody titers. In sharp contrast, boosting the primed huCD4 *Tg* mice (which express huCD4 on both T and B cells) with soluble gp120 resulted in lower secondary antibody titers than in controls. The response to an irrelevant antigen, HEL, was also reduced in the gp120 -primed and boosted huCD4 Tg mice. Furthermore, on day 6 after a single bolus of gp120, the number of peripheral T cells and B cells in immunized huCD4 Tg decreased to 50% of the control levels. Moreover, compared to the control groups, the splenocytes from gp120-pretreated immunized huCD4 Tg had a lower level of CD3+ T cells and underwent extensive apoptosis after anti-CD3 treatment. These in *vivo* results were consistent with the *in vitro* findings: Crosslinking of huCD4 on the spleen lymphocytes of huCD4 Tg mice using

$rgp120_{SF2}$ and anti-gp120 antibody not only sensitized T cells for apoptosis, but also induced apoptosis *per se*. Thus, precautions should be taken when employing HIV envelope gp120 as one of the HIV vaccine components. Tolerogenic therapies should be considered when treating HIV infected subjects in this manner.

Materials and Methods

Mice -- BALB/cByJ and CB6 F1 mice were purchased from the Jackson Laboratories (Bar Harbor, ME) at 6-10 weeks of age, and housed in pathogen-free, microisolater cages. Line 313 huCD4 Tg mice were obtained from Dr. Terri Finkel, Denver, CO). These transgenics were originally produced by Dr. Richard Flavell by injecting a huCD4 transgene into fertilized eggs and were maintained by repeated backcrosses on the C57Bl/6 background [17]. The F_1 offspring between huCD4 Tg mice and BALB/cByJ are produced in our animal facility by crossbreeding female BALB/cByJ with male huCD4 Tg mice to yield huCD4 expressing mice histocompatible with CB6 F1 mice. The huCD4 molecule was shown to be functional in calcium signal transduction and in overcoming the block in positive selection induced by *in vivo* injection of mAbs to the endogenous mouse CD4 [17]. Since in these transgenic mice, expression of huCD4 is driven by CD2 regulatory elements, both B and T cells express huCD4. Approximately, 85% of splenic cells from Line 313 huCD4 transgenic mice and more than 50% of spleen cells from the F_1 offspring between huCD4 Tg mice and BALB/cByJ expressed the huCD4 receptor on their surface. Recombinant wild type gp120 ($rgp120_{SF2}$), from Dr. K. Steimer (Chiron Corporation, Emeryville, CA), binds to the huCD4 molecules in a dose-dependent manner and competes with huCD4 mAb (Leu-3a; Becton Dickinson, Mountain View, CA) for huCD4 binding.

Cell Culture

RPMI 1640 medium (GIBCO-BRL, Gaithersburg, MD) supplemented with heat-inactivated 5% fetal calf serum (Hyclone, Logan, UT), 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, MEM nonessential amino acids, and 1 mM sodium pyruvate was used.

Antibodies

The following antibodies were purchased from PharMingen (San Diego, CA): FITC-labeled anti-hamster IgG, PE-labeled anti-mouse CD3, biotin-labeled anti-mouse CD19 and anti-Fas antibody Jo2) FITC- labeled mouse anti-human CD4 mAb (FITC-Leu-3a) was obtained from Becton Dickinson (View Mountain, CA). The following biotin- or FITC- labeled antibodies were purified in our lab by standard protocols: anti-mouse CD3 (145.2C11), anti-mouse CD4 (GK1.5), anti-mouse CD8 (53-6.72) and anti-mouse CD45R (RA36B2, B220). Anti-gp120 antibodies used for huCD4 crosslinking were obtained as follows: human monoclonal anti-gp120 antibodies directed against gp120 C-terminal peptide, (450-30D 100,100,1,1; abbreviated "450-30")) and against the V3 loop (694/98-D 100,100,10,1; abbreviated "694/98") were from Dr. Susan Zolla-Pazner (VA Medical Center, NY); sheep anti-gp120 antibody (6205) directed against the 15 C-terminal residues was obtained from International Enzymes, Inc. (Fallbrook, CA); human HIV-1 gp120 monoclonal antibodies (F105) [Dr. M. Posner, refs. 18-21] and (AD3) [Drs. K. Ugen and D. Weiner, ref. 22], as well as goat polyclonal HIV-1SF2 gp120 antibodies raised against glycosylated and non-glycosylated gp120 [Dr. K. Steimer, refs. 23-27] were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. Mouse polyclonal HIV-1_{SF2} gp120 antisera were produced in our lab by immunization of BALB/cJ mice with HIV-1_{SF2} gp120 in CFA intradermally and boosting twice with HIV-1_{SF2} gp120 in IFA intramuscularly after 2 weeks and 1 month of primary immunization, respectively. The antibody titer was $>1/10^5$ as determined by ELISA using rgp120 coated plates (1 μg/ml in Tris coating buffer, pH 9.0).

In vitro crosslinking huCD4 by gp120 and anti-gp120 followed by anti-CD3 activation

Viable splenocytes from Line 313 huCD4 Tg were incubated (5 x 10^6/ml) with $rgp120_{SF2}$ (20 (g/ml) on ice for 30 min., washed twice, and reincubated with various anti-gp120 antibodies (2 (g/ml or 1:1000 dilution) at 37°C for 45 min. While an aliquot of the cells was checked for surface levels of Fas expression by flow cytometry, 1 x 106 cells were transferred onto anti-CD3 antibody (145.2C11)-precoated 96 well plates in a 200 (l volume and incubated at 37°C, 5% CO_2 for further

24-72 hr. The cells were then harvested and assayed for apoptosis by DNA content analysis, as described below [28].

Immunization protocol and intravenous administration of gp120

BALB/cJ mice and Line 313 huCD4 transgenic mice were immunized intradermally with 20 μg HIV-1_{SF2} rgp120 emulsified in CFA and boosted intramuscularly with 20 μg rgp120 in IFA 9-12 days later. Ten days after boosting, when high titers of anti gp120 sera were detectable, a single dose of 1 μg of rgp120 was administered intravenously into the immunized animals, as well as animals that had not received rgp120 immunization. The percentages of human CD4+, mouse CD3+, CD8+ and B220+ cells in the peripheral blood were followed using dual color flow cytometry described below. In some experiments, the spleens were harvested within 9 days after gp120 iv injection and the percentages of splenic CD3+ T cells were determined by flow cytometry. The apoptotic cells in freshly harvested spleens were assessed by *in situ* terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling staining (TUNEL). Spontaneous apoptosis and anti-CD3 activation-induced apoptosis was measured after anti-CD3 *in vitro* treatment for 24-72 hr. To search for depletion of antigen-specific T cells by repeated gp120 iv injection, the F_1 offspring between huCD4 Tg and BALB/cByJ mice, as well as CB6 F1 control mice, were immunized intradermally with 20 μg HIV-l_{SF2} rgp120 and 20 μg hen egg-white lysozyme (HEL, Sigma Chemical Co., St. Louis, MO) emulsified in CFA. Three weeks after primary immunization, the animals were boosted intravenously with 1 μg $rgp120_{SF2}$ and 1 μg HEL in PBS, and the iv injection was repeated three times at 10 day intervals. One week after each iv injection, the CD3+ T cells were determined by flow cytometric analysis and the gp120 and HEL specific IgG responses were measured by ELISA by coating plates with 1 μg/ml gp120 or 1 μg/ml HEL, respectively. Antibody titers were determined using CA-Cricket Graph software and were expressed as the serum dilution that would bring the OD to pre-immunization levels ($OD_{405} \simeq 0.04$), assuming parallelism of curves. In other experiments, gp120-primed mice were sacrificed on day 1, 4, 7, 11, and 20 after a single bolus of gp120

iv, and lymph nodes and spleens were harvested,and determinations made of cell phenotypes, spontaneous apoptosis and anti-CD3 stimulation index.

Flow cytometric analysis

The surface level of Fas expression on the splenocytes from Line 313 huCD4 transgenic mice after huCD4 crosslinking was measured by staining cells with hamster anti-Fas antibody (Jo2), followed by FITC-labeled anti-hamster IgG. To determine the percentage of peripheral CD3+ T cells, blood was removed from the retroorbital plexus. White blood cells were prepared by lysing red blood cells with Tris-buffered ammonium chloride buffer (pH 7.2). After washing with PBS, the cells were stained with FITC-labeled Leu-3a, or anti-B220, or anti-CD8 antibody plus biotin-labeled anti-mouse CD3 antibody, followed by Streptavidin-PE staining. The same procedure was applied to spleens for measurement of CD3+ T cells. To measure Annexin V positive T cells, lymph node and spleen cells were washed twice with PBS and resuspended in binding buffer (10 mM Hepes/NaOH, pH 7.4,140 mM NaCl, 2.5 mM $CaCl_2$) [41]. To the cell resuspension was added fluorescein labeled Annexin V (R&D SYSTEMS, Inc., Minneapolis, MN) and biotin-labeled anti-CD3 antibody, followed by Streptavidin-PE staining. The FITC and PE fluorescence analysis were performed by CELLQuest software in FACScan flow cytometry (Becton Dickinson).

Apoptosis analysis

The percentage of cells undergoing apoptosis was quantitated by a flow cytometric method described earlier [28]. Briefly, cells (1×10^6) were fixed in 70% ethanol for 1 hour at 4 C. The cells were then washed and resuspended in 1 ml PBS, to which 1 µl RNase solution (10 mg/ml in PBS) was added and incubated at 37°C for 1 hour. Following the addition of 5 µl of propidium iodide (PI, 10 mg/ml in PBS), the PI fluorescence of individual cells was measured using flow cytometry. Cell debris and clumps were excluded by gating for single cells by forward and side light scatter and by FL-2 area vs. FL-2 width. A distinct cell cycle region of apoptosis (A_0) could be identified below the G_0/G_1 diploid peak and the percentage of cells in the A_0 region was quantitated.

Statistical Analysis

The difference among different treatment groups was analyzed by one-way ANOVA (StatView, BrainPower, Inc., Calabasas, CA).

RESULTS

In vitro induction of apoptosis by gp120 crosslinking of huCD4

We hypothesized that gp120 crosslinking of huCD4 on splenic cells from huCD4 transgenic mice would induce apoptosis or prime for apoptosis despite the fact that these cells cannot be infected by HIV [34] due to the lack of a necessary cofactor (such as fusin) in mice [35]. We cultured splenocytes from Line 313 huCD4 transgenic mice with $rgp120_{SF2}$, and then crosslinked huCD4 by the addition of murine hyperimmune anti-gp120 antibody, and finally stimulated aliquots of these cells with anti-CD3 antibody. Data from three sets of 24 hr *in vitro* apoptosis induction experiments showed that crosslinking huCD4 by gp120 and anti-gp120 antibody prior to TCR ligation primed huCD4 Tg splenocytes for anti-CD3 activation-induced apoptosis. In contrast, anti-CD3 antibody alone reduced spontaneous apoptotic cell death. This phenomenon of reduction of apoptosis in unprimed T cells presumably reflects cell cycle entry induced by anti-CD3 activation. Furthermore, crosslinking huCD4 by gp120 and anti-gp120 antibody *per se* induced apoptosis in the huCD4 Tg splenocytes, though to a lesser extent than that with additional anti-CD3 treatment. Neither gp120 alone nor anti-gp120 antibody alone had any effect on the priming and apoptosis induction, suggesting that anti-gp120 antibody was required for huCD4 crosslinking-mediated apoptosis induction *in vitro*.

Crosslinking huCD4 on spleencells of these transgenic mice via gp120 and anti-gp120 antibody modestly upregulated surface levels of Fas expression (Table VIII), as shown by others [10, 13, 14]. Furthermore, the induction of apoptosis and upregulation of Fas expression by huCD4 crosslinking is not dependent on the specificity of anti-gp120 antibody. Thus, we tested a variety of anti-gp120 antibodies for huCD4 crosslinking, either mAbs raised against a number of gp120 antigenic domains or polyclonal antibodies. The results on induction of apoptosis and

upregulation of Fas expression using different kinds of anti-gp120 antibodies for CD4 crosslinking are summarized in Table VIII.

gp120 Induces Acute Loss of CD3+ T cells in Immunized huCD4 Transgenic Mice

A paradox in HIV pathogenesis is that the virus appears to cause AIDS after the onset of antiviral immunity [11]. Wang et al [15] reported that injection into huCD4 transgenic mice of HIV-gp120 and subsequent gp120-specific antibodies from AIDS patients (passive transfer of immunity) induced massive long-lasting T lymphocyte deletion. To investigate if an ongoing immune response to gp120 (active immunity) could crosslink huCD4 and lead to T cell depletion upon exposure to HIV-gp120, we administered $gp120_{SF2}$ intravenously into $gp120_{SF2}$ immunized huCD4 transgenic mice.

The time course of CD3+ T cell loss in an *in vivo* experiment was analyzed Though the number of peripheral CD3+ T cells varied in individual mice, there was a drop of peripheral CD3+ T cells only in immunized huCD4 Tg mice after a single i.v. injection of gp120. None of the other three control groups showed a drop of CD3+ T cells. This CD3+ T cell loss occurred acutely, reached its peak (50% of the pre-injection level) on day 6 after i.v. gp120 injection and then gradually recovered. Although we did observe CD3+ T cell depletion for more than two weeks in individual animals , the CD3 + T cell drop in the majority returned to its preinjection level within two weeks.

Table VIII. Apoptosis induction and Fas upregulation on splenocytes of huCD4 transgenic mice by CD4 crosslinking using various anti-gp120 antibodies directed against a number of g120 antigenic domains

Anti-gp120 antibodies	Specificity & Species	Apoptosis Induction with anti-CD3 (%)	Fas Upregulation % of Control
450-30	C5 domain (PTKAKRR) human mAb	56%	13%
594/98	V3 loop Human mAb	64%	16%
F105	Conformational epitope human mAb	71%	12%
AD3	First 204 amino acids murine mAb	70%	15%
6205	C5 domain (aa 497-511) sheep Ab	74%	13%
Anti-gp120 ab #6	Glycosylated gp120 goat polyclonal Ab	67%	10%
Anti-gp120 ab #7	Non-glycosylated gp120 goat polyclonal Ab	64%	13%
Anti-gp120 ab #8	$Rgp120_{SF2}$ murine polyclonal Ab	75%	2%
Medium Control no CD4 crosslink. no anti-CD3		29%	0%
Anti-CD3-Control no CD4 crosslink.		20%	

Splenocytes from huCD4 transgenic mice were treated and analyzed as described. Apoptosis induction with anti-CD3 (%) refers to the percentages of apoptotic cells after crosslinking of huCD4 crosslinking with anti gp120 antibodies and after 24 hr of treatment with anti-CD3. Fas upregulation by huCD4 crosslinking for 45 min. was calculated as the % increase in median fluorescent channel over medium control.

The mean percentages of peripheral blood CD3+ T cells on day 0 and day 6 after i.v. gp120 injection from all i*n vivo* experiments were compared. On day 6, injection of gp120 induced significant peripheral CD3+ T cell loss in immunized huCD4 Tg, compared to the other three groups.

The first bolus of gp120 induced a significant, though transient, loss of peripheral CD3+ T cells in huCD4 transgenic mice, but not in CB6 F1 control mice. Surprisingly, repeated gp120 iv injections afterwards were not able to produce a state

of long-lasting T cell loss, neither to induce the T cells to decline again after recovery from the first drop, though a slight lower level of CD3+ T cells were constantly observed after gp120 injections in the huCD4 transgenic mice than in the controls. Interestingly, gp120 i.v. injection also resulted in a loss of peripheral blood CD19+ B cells which express huCD4 driven by CD2 promoter but not the CD3-/CD19-cells in the gp120-immunized huCD4 transgenic mice, in the same pattern as the loss of CD3+ T cells.

T cell depletion via apoptosis is not restricted to peripheral blood, but also occurs in spleen and lymph nodes

To investigate if T cells in spleen and lymph node are also deleted in immunized huCD4 transgenic mice receiving gp120 iv injection, we harvested spleens and lymph nodes 1 - 20 days after the first gp120 i.v. injection and measured CD3+ T cells by flow cytometry. The numbers of splenic CD3+ T cells in all gp120-immunized and -pretreated huCD4 Tg were decreased to 50-75% of those in control groups. Moreover, lower levels of CD3+ T cells were also observed in the lymph nodes from gp120 - immunized and -pretreated huCD4 Tg. The decrease in CD3+ T cells in spleens and lymph nodes suggested that the T cell drop was not restricted to peripheral blood. Furthermore, as measured in a TUNEL assay, while no difference from control groups was noted in some gp120-pretreated immunized huCD4 Tg, a slightl increase (by 3-5%) in apoptotic cell death was observed in freshly harvested spleens from other gp120-pretreated immunized huCD4 Tg, as shown by others [16]. In addition, when fluorescein labeled Annexin V was employed to detect phosphatidylserine expression on early apoptotic cells [41], a higher percentage of Annexin V positive T cells was observed in the spleens and the lymph nodes from gp120-immunized huCD4 transgenic mice than in the organs of control mice, indicating that gp120-induced apoptosis occurs in peripheral lymphoid organs as well as blood. Taken together, these results suggest that a higher number of T cells in the gp120-immunized huCD4 transgenic mice undergo apoptosis after receiving gp120 iv injection.

gp120 injections lead to a lower secondary antibody titers in primarily immunized huCD4 transgenic mice, presumably by depletion of antigen-specific T and B cells

To further confirm that crosslinking *in vivo* of huCD4 by gp120/anti-gp120 binding causes depletion and/or sensitizes huCD4-expressing T and/or B cells for activation-induced apoptosis, we injected gp120 and an irrelevant antigen (HEL) i.v. into huCD4 Tg mice and CB6 F1 mice that had received primarily immunization with gp120 and HEL in CFA. We then measured the secondary antibody titers against gp120 and HEL. Boosting with gp120 not only boosts specific T and B cells for secondary responses, but the gp120 can also bind to huCD4 receptors to prime for apoptosis on all huCD4+ cells. We used the response to HEL as an irrelevant control response although HEL-specific T and B cells would be expected to bind gp120 to their huCD4 receptors like gp120 specific cells. The results showed that huCD4 Tg mice primed with soluble gp120 had lower secondary titers than did controls, and the response to an irrelevant antigen, HEL, was also reduced in the gp120 – primed/boosted huCD4 Tg mice. These results indirectly support our hypothesis that gp120 injection depletes a population of antigen-specific T cells and/or B cells.

TCR ligation induces further apoptosis in the spleens of immunized huCD4 transgenic mice

As mentioned above, in immunized huCD4 Tg mice, the total CD3+ T cell population was depleted by 50%. Numbers of CD3+ cells then recovered to pre-injection levels within two weeks after a bolus of gp120 injection. To test if the undeleted / recovered CD3+ T cells were primed for apoptosis by *in vivo* huCD4 crosslinking, we harvested spleens after a single bolus of gp120 and assayed *in vitro* the apoptosis stimulated by anti-CD3 antibody. Anti-CD3 treatment resulted in lower stimulation in spleen cells from gp120 - immunized and -pretreated huCD4 Tg compared to CB6 F1 control mice. Anti-CD3 treatment also increased the percentage of apoptotic cells compared to the medium control treatment only in immunized huCD4 Tg mice receiving a bolus injection of gp120 . These *ex vivo* results indicated that the undeleted / recovered CD3+ T cells in the gp120-pretreated immunized huCD4 Tg mice were also primed for apoptosis by crosslinking of huCD4 *in vivo* .

DISCUSSION

Efforts are currently underway to elucidate the mechanisms responsible for AIDS pathogenesis and to establish a protective vaccine for HIV. The precursor envelope glycoprotein of HIV, gp160, and mature proteins, gp120 and gp41, have been considered to be important if not essential as vaccine components, because epitopes of these proteins induce both antibody and cytotoxic T cell responses in man. Although usually viewed as potentially protective, the role of humoral immune responses to viral envelope gp120 in HIV pathogenesis was investigated in the current study. Our findings , as well as those of others [11, 14-16, 29, 30], suggest that endogenous anti-gp120 antibody in AIDS patients' serum may actually promote rather than neutralizing and inhibitng HIV pathogenesis. Therefore, to avoid provoking even greater T cell depletion in AIDS, tolerogenic therapies should be considered in HIV vaccine design . This concept is supported by the results of Finkel *et al.* [16], who used huCD4 and HIV gp120 double transgenic mice to address the role of anti-gp120 antibody in T cell depletion. Their findings, that antibodies crosslinking huCD4/gp120 complexes are a determinant for the outcome of the T cell responses to stimuli *in vitro* and *in vivo*, are consistent with the present results.

Here, we followed the numbers of CD3+ T cells in human CD4 transgenic mice in vivo after immunization and a single intravenous exposure to soluble gp120. In such mice, a bolus of gp120 led to rapid depletion of CD3+ T cells in the periphery and in the spleen of gp120 immunized huCD4 transgenic mice; non-immune transgenic or immunized non-transgenic mice were unaffected by this treatment. We measured CD3 T cells instead of human CD4 expressing cells since, in preliminary studies, we found that membrane expression of huCD4 was either downregulated or blocked by gp120:anti-gp120 binding. Expression of huCD4 is driven in these mice by the CD2 promoter, which results in expression in both T and B cells (over 80% of splenocytes). Our results indicate that huCD4-expressing B cells may also be depleted *in vivo*. In humans, a very small number of B cells (0.1-1%) express CD4 molecules annd their function in human is still unclear, as is their fate in HIV infection. More importantly, the present results suggest that huCD4 crosslinking-

transmitted death signal might not necessarily require association with TCR/CD3 signaling pathway and that, under appropriate circumstances, huCD4 crosslinking is enough to send the death signal and induce the cells to die.

Because we cannot exclude Fas involvement in huCD4 crosslinking-mediated apoptosis, it is important to know the threshold of Fas expression in apoptosis induction. While there is a higher percentage of Annexin V positive T cells in the lymph nodes and spleens from gp120-pretreated immunized huCD4 transgenic mice than those in the control mice, we have not detected a significant increase in apoptotic cells in vivo in these tissues using in situ TUNEL staining. Annexin V binds to phosphatidylserine, which is translocated from the inner side of the plasma membrane to the outer layer and becomes exposed at the external surface on early apoptotic cells [41]. This event occurs well before the DNA fragmentation as measured by in situ TUNEL staining, therefore, it is possible that rapid phagocytosis of early stage apoptotic T cells may be occurring so that an increased level of apoptotic cells may be evanescent.

Experiments in which repeated gp120 injections are given to the immunized huCD4 transgenic mice suggest that at least some antigen specific T cells and B cells have been depleted by gp120/anti-gp120 antibody crosslinking huCD4 via apoptosis. However, to our surprise, multiple gp120 injections did not induce a long-lasting T cell loss from the periphery, though the splenocytes from these mice were still primed for TCR activation-induced apoptosis *in vitro*. Given the fact that huCD4 transgenic mice produced a large amount of anti-gp120 antibodies after the first bolus of gp120, circulating in the bloodstream, gp120 injected thereafter may not be able to compete for binding to huCD4 molecules. tthe relatively rapid recovery of CD3 T cells *in vivo* may reflect the small amount of available gp120 when delivered to the bloodstream of immunized mice as a bolus, in contrast to the small but steady production of viral gp120 by HIV in infected individuals. Recent studies [16] with non-tolerant gp120 transgenic mice are encouraging for the validity of this model. Transgenic mice in which production of gp120 can be controlled by an inducible promoter (to avoid partial tolerance seen in other work [16]) would be helpful in this direction.

In summary, our studies demonstrate that an ongoing humoral immune response to gp120 in huCD4 transgenic mice can participate in priming T cells to die upon exposure to gp120. While transient, these primed T cells remain hypersensitive to activation-induced death in vitro by antiCD3 crosslinking, and undergo apoptosis *in vivo* when exposed to a specific antigen that can bind to their TCR. We believe this system will be useful for evaluating one of the fundamental processes underlying HIV pathogenesis. Using the present invention, it will be possible to test potential immune modulation therapies to reverse this sensitivity to apoptosis by inducing tolerance to gp120 epitopes [40].

References

(1) Ameisen, J. C. *et al.*, Immunol. Today 1991. 12:102; (2) Starcich, B. R. *et al.*, Cell 1986. 45:637; (3) Abacioglu *et al., supra;* (4) Modrow, S.*et al.*, J. Virol. 1987. 61:570; (5) Willey, R. L.*et al.*, Proc. Natl. Acad. Sci. USA 1986. 83:5038; (6) Weiss, R. A.*et al.*, Nature 1985. 316:69; (7) Katz, D. H., AIDS Res. Hum. Retro. 1993. 9:489; (8) Golding, H. *et al.*, J. Exp. Med. 1988. 167:914; (9) Kion, T. A. *et al.*, Science 1991. 253:1138. (10) Desbarats *et al. supra;* (11) Banda *et al., supra* [J. Exp. Med. 1992. 176:1099; (12) Newell *et al., supra* .[Nature 1990. 347:286; (13) Oyaizu *et al.*, 1994, *supra* [Blood 1994. 84:2622; (14/15) Wang *et al., supra;* (16) Finco, O. *et al.*, Eur. J. Immunol. 1997. 27:1319; (17) Paterson, R. K. *et al.*, J. Immunol. 1994. 153:3491; (18) Posner, M. R. *et al.*, Acquired Immune Defic. Syndr. 1993. 6:7; (19) Cavacini, L. A. *et al.*, J. Acquired Immune Defic. Syndr. 1993. 6:353; (20) Posner, M. R. *et al.*, Hybridoma 1987. 6:611; (21) Posner, M. R. *et al.*, J. Immunol. 1991. 146:4325; (22) Ugen, K. E. *et al.*, In: Vacines 93, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. 1993. p. 215; (23) Haigwood, N. L. *et al.*, AIDS Res. Hum. Retro. 1990. 6:855; (24) Steimer, K. S. *et al.*, In: Vacines 88, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. 1988. p. 347; (25) Levy, J. A. *et al.*, Science 1984. 225:840; (26) Sanchez-Pescador *et al., supra;* (27) Haigwood, N. L. *et al.*,, In: Vacines 90, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. 1990. p. 313; (28) Ezhevsky, S. A. *et al.*, Mol. Biol. Cell 1996. 7:553; (29) Goronzy, J.*et al.*, J. Exp. Med. 1986. 164:911; (30) Jamali, I.*et al.*, J. Immunol. 1992. 148:1613; (31) Muro-Cacho, C. A. *et al.*, J. Immunol. 1995. 154:5555; (32) Chun, T.-W. *et al.*, Nature 1997. 387:183; (33) Harper, M. E. *et al.*, Proc. Natl. Acad. Sci. USA 1986. 83:772; (34) Lores, P. *et al.*, AIDS Res. Hum. Retro. 1992. 8:2063; (35) Feng, Y. *et al.*, Science 1996. 272:872; (36) Scott, D. W. *et al.*, J. Immunol. 1996. 156:2352; (37) Lanzavecchia, A., J. Exp. Med. 1995. 181:1945; (38) Salemi, S. *et al.*, J. Exp. Med. 1995. 181:2253; (39) Siliciano, R. F. *et al.*, Cell 1988. 54:561; (40) Zambidis, E. T. *et al.*, Mol. Med. 1997. 3:212; (41) Vermes, I. *et al.*, J. Immulnol. Meth. 1995. 184:39.

EXAMPLE VII

Induction of Immune Tolerance to Foreign Immunogenic Epitopes via Retroviral-Mediated Expression of Foreign Protein: the IgG Scaffold is Important for Induction and Maintenance of Humoral Immunological Tolerance

Results described above showed that recipients of bone marrow or of LPS-stimulated B-cell blasts, both of which were retrovirally gene-transferred with an immunodominant peptide in-frame to the V region of a murine IgG H chain, were rendered profoundly unresponsive to that epitope. To further investigate whether tolerance to larger molecules can be achieved via this approach and whether the IgG scaffold is important for induction and maintenance of immunological tolerance, we engineered two retroviral constructs (MBAE-1-102 and MBAE-1-102-IgG). The first of these included the DNA encoding the p1-102 peptide of bacteriophage λ. The second had DNA encoding that peptide fused to the murine Ig γ chain DNA such that the peptide was expressed at the N-terminus of the H chain. These vectors were used for gene transfer.

Specificity of p1-102 humoral tolerance in genetically tolerized bone marrow recipients was examined. CB6 F_1 mice were sublethally irradiated with 400 rads and injected with mock-transduced or 1-102 -IgG gene-transduced bone marrow cells. Mice were primed and boosted with p1-102 and HEL in CFA. Antibody responses were measured in ELISA by coating plates with 50 μg/ml synthetic peptides (peptides 12-26, 73-88 or 55-69). The titers were determined by using CA-Cricket graph software and expressed as the dilution which brings the OD_{405} to the pre-immune level. Each experiment had 3-4 mice per group. The efficacy in induction and maintenance of tolerance by p1-102 and p1-102-IgG gene transfer in bone marrow recipients was also examined. CB6 F_1 mice were sublethally irradiated as above and injected with mock-transduced or p1-102 transduced or p1-102 -IgG gene-transduced bone marrow cells. Mice were later primed and boosted with p1-102 and HEL in CFA. Antibody responses were assayed and analyzed as above.

The results showed that recipients of bone marrow cells or peripheral B cells that had been gene-transferred with MBAE-1-102-IgG were specifically

hyporesponsive to the p1-102 protein (and this occurred in a strain-specific manner). That is, Balb/c and C57Bl/6 mice recognize epitopes contained in residues 12-26 and 73-88, respectively, whereas F_1 hybrid mice between these strains recognize epitopes at both sites. Gene-transfer produced tolerance in F_1 mice to the whole p1-102 protein, as well as to the major determinants. No "epitope spreading" to minor epitopes was observed. The results suggest that the self IgG scaffold is necessary for long-lasting unresponsiveness because recipients of p1-102-IgG fusion protein construct remained tolerant to secondary challenge whereas controls given cells transfected with p1-102 construct (not fused to the IgG) regained responsiveness. These results demonstrated that the host can then present the relevant epitopes in a MHC-haplotype-specific manner to the immune system and induce profound tolerance. This results is directly applicable to treatment of autoimmune diseases, as well as for creating a receptive environment for foreign or otherwise immunologically "unacceptable" proteins to be administered in the context of gene therapy.

The references cited above are all incorporated by reference herein, whether specifically incorporated or not.

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims

WHAT IS CLAIMED IS:

1. A fusion immunoglobulin (Ig) heavy (H) chain protein comprising a mammalian Ig H chain fused in frame in its N-terminus to one or more HIV gp120 epitopes, wherein said fusion Ig H chain is tolerogenic in a host with respect to said gp120 epitopes.

2. A fusion Ig H chain protein according to claim 1, wherein said gp120 epitope is fused to said mammalian Ig H chain at its N-terminal region.

3. A fusion Ig protein comprising two Ig H chains and two Ig light (L) chains, wherein at least of said H chains is a fusion Ig H chain according to claim 1.

4. A fusion Ig protein according to claim 3, wherein both of said H chains are Ig H chains according to claim 1.

5. A fusion Ig protein according to claim 1, wherein said H chain is a Ig γ chain.

6. A fusion Ig protein according to claim 5, wherein said H chain is a human Ig γ_3 chain

7. A fusion Ig H chain according to claim 1, wherein said one or more gp120 epitopes comprises a full length gp120 protein.

8. A fusion Ig H chain protein according to claim 1, wherein said mammalian Ig is human Ig.

9. A fusion Ig H chain according to claim 1, wherein said one or more gp120 epitopes comprises a C1 region peptide, a V3 loop peptide or a C5 region peptide.

10. A fusion Ig H chain according to claim 1, wherein said one or more gp120 epitopes is a B cell epitope which is a peptide selected from the group

consisting of VPVWKEATTTLFCASDAKAY (SEQ ID NO:2), EVHNVWATHACVPTD (SEQ ID NO:3), YDTEVHNVWA (SEQ ID NO:4), PQEVVLVNVT (SEQ ID NO:5), PQEVVLVNVTENFDMWKNDM (SEQ ID NO:6), PNNNTRKSIR (SEQ ID NO:7), NNNTRKRIRIQRGPGR (SEQ ID NO:8), RKSIR (SEQ ID NO:9), IQRGPGRAFV (SEQ ID NO:10), GRAFVTIGKI (SEQ ID NO:11), PGRAFY (SEQ ID NO:12), NTRKSIRIQRGPGRAFVTIG (SEQ ID NO:13), PNNNTRKSIRIQRGPGRAFVTIGKIGNMRQAHC (SEQ ID NO:14), NNTRKSIRIQRG (SEQ ID NO:15), NKRKRIHIGPGRAFYTTKNIIGTIC (SEQ ID NO:16), RKSIRIQRGPGRAFV (SEQ ID NO:17), IRIQRGPGR (SEQ ID NO:18), KRIRIQRGPGRAFVTIG (SEQ ID NO:19), QRGPGRAF (SEQ ID NO:20), RGPGRAFV (SEQ ID NO:21), RKRIHIGPGRAFYTT (SEQ ID NO:22), RGPGRAFVTIG (SEQ ID NO:23), SISGPGRAFYTG (SEQ ID NO:24), KRIHI (SEQ ID NO:25), KRIHIGP (SEQ ID NO:26), IHIGPGR (SEQ ID NO:27), HIGPGR (SEQ ID NO:28), HIGPGRA (SEQ ID NO:29), HIGP (SEQ ID NO:30), RIHIGPGRAFYTTG (SEQ ID NO:31), RIQRGPGRAF (SEQ ID NO:32), IQRGPGRAFV (SEQ ID NO:10), IQRGPGRAF (SEQ ID NO:33), IRIQRGPGRAFVTI (SEQ ID NO:34), RGPGRAFVTIGKIG (SEQ ID NO:35), QRGPGRA (SEQ ID NO:36), IXXGPGRA (SEQ ID NO:37), IGPGR (SEQ ID NO:38), GPGR (SEQ ID NO:39), GPXR (SEQ ID NO:40), GPGRAF (SEQ ID NO:41), RIHIG (SEQ ID NO:42), HIGPGRAF (SEQ ID NO:43), GRAF (SEQ ID NO:44), GGGDMRDNWRSELYKYKVVK (SEQ ID NO:45), KYKVVKIEPLGVAPTKAKRR (SEQ ID NO:46), LGVAPTKAKR (SEQ ID NO:47), GGDMRDNWRSELYKYKVVKI (SEQ ID NO:48), IEPLGVAPTK (SEQ ID NO:49), RRVVQRE (SEQ ID NO:50), PTKAKRR (SEQ ID NO:51) and VVQREKR (SEQ ID NO:52).

11. A fusion Ig H chain according to claim 1, wherein said one or more gp120 epitopes comprises a T cell epitope which is a peptide selected from the group consisting of EQLWVTVYYGVPV (SEQ ID NO:53), VYYGVPVWKEA (SEQ ID NO:54), GVPVWKEATTLFC (SEQ ID NO:55), AHKVWATHACV (SEQ ID NO:56), NVWATHACVPTD (SEQ ID NO:57), CVPTNPVPQEVV (SEQ ID NO:58), VEQMHEDIISLW (SEQ ID NO:59), EQMHEDIISLWDQ (SEQ ID NO:60), EQMHEDIISLWDQSL (SEQ ID NO:61), HEDIISLWDQSLK (SEQ ID NO:62), VTVYYGVPVWKEATTTLFC (SEQ ID NO:63), VVLVNVTENFNM (SEQ ID NO:64), SLKPCVKLTPLCY (SEQ ID NO:65), CTRPNNNTRKSIRIQRGPGY (SEQ ID NO:66),

NTRKSIRIQRGPGR (SEQ ID NO:67), EQRGPGRAFVTIGKI (SEQ ID NO:68), RIQRGPGRAFVTIGK (SEQ ID NO:69), RIHIGPGRAFYTTKN (SEQ ID NO:70), GRAFVTIGKIGNMRQ (SEQ ID NO:71), QRGPGRAFVTIGKIGNMRQAH (SEQ ID NO:72), VGKAMYAPPISGQIR (SEQ ID NO:73), GNSNNESEIFRPGGG (SEQ ID NO:74), FRPGGGDMRDNWRSEL (SEQ ID NO:75), DMRDNWRSELYKYKV (SEQ ID NO:76), RDNWRSELYKYKVVK (SEQ ID NO:77), CKYKVVKIEPLGVAPT (SEQ ID NO:78), YKYKVVKIEPLGVAP (SEQ ID NO:79), KVVKIEPLGVAPTKAKRRVVQREKRC (SEQ ID NO:80), ITLPCRIKQIINMWQEVGKAMYAPPISGQIRC (SEQ ID NO:81), and ELYKYKVVKIEPLGVAPTKAKRRVVQREKR (SEQ ID NO:82).

12. A DNA molecule comprising a nucleotide sequence encoding a fusion Ig H chain according to claim 1.

13. A DNA molecule comprising a nucleotide sequence encoding a fusion Ig H chain according to any of claims 5-10.

14. An expression vector which expresses a product useful for inducing and maintaining immunological tolerance to one or more epitopes of HIV gp120 protein in a subject, comprising:

(a) a DNA molecule according to claim 1, operably linked to

(b) transcriptional and translational control regions operable in a hematopoietic cell or lymphoid cell of said subject.

15. An expression vector which expresses a product useful for inducing and maintaining immunological tolerance to one or more epitopes of HIV gp120 protein in a subject, comprising:

(a) a DNA molecule according to any of claims 2 or 5-10, operably linked to

(b) transcriptional and translational control regions operable in a hematopoietic cell or lymphoid cell of said subject.

16. A vector according to claim 13 which is a retroviral vector.

17. A vector according to claim 13, wherein the transcriptional and translational control regions provide for constitutive expression of the DNA sequence in lymphoid cells.

18. A hemopoietic or lymphoid cell transformed by a vector according to claim 13, which cell stably expresses said fusion Ig.

19. A hemopoietic or lymphoid cell transformed by a vector according to claim 14, which cell stably expresses said fusion Ig.

20. A hemopoietic or lymphoid cell transformed by a vector according to claim 15, which cell stably expresses said fusion Ig.

21. A human bone marrow cell transformed by a vector according to claim 13, which cell stably expresses said fusion Ig.

22. A cell according to claim 17 which is a B lymphocyte.

23. A B lymphocyte according to claim 21 which has been activated in culture.

24. A pharmaceutical composition comprising:

(a) a tolerogenic amount of a fusion Ig molecule having a fusion Ig H chain according to claim 1; and

(b)a pharmaceutically acceptable carrier or excipient for parenteral administration.

25. A pharmaceutical composition according to claim 21, wherein the Ig is an isologous IgG molecule.

26. A pharmaceutical composition comprising:

(a) a tolerogenic amount of a fusion Ig molecule having a fusion Ig H chain according to any of claims 2, or 5-10; and

(b) a pharmaceutically acceptable carrier or excipient for parenteral administration.

27. A pharmaceutical composition comprising:

(a) a tolerogenic amount of a fusion Ig molecule according to claim 3 or 4; and

(b) a pharmaceutically acceptable carrier or excipient for parenteral administration.

28. A method for immunologically tolerizing a subject to one or more HIV gp120 epitopes comprising administering to said subject an effective amount of a pharmaceutical composition according to claim 23.

29. A method for immunologically tolerizing a subject to one or more HIV gp120 epitopes comprising administering to said subject an effective amount of a pharmaceutical composition according to claim 24.

30. A method for immunologically tolerizing a subject to one or more HIV gp120 epitopes comprising administering to said subject an effective amount of a pharmaceutical composition according to claim 25.

31. A method for immunologically tolerizing a subject to one or more HIV gp120 epitopes comprising administering to said subject an effective amount of a pharmaceutical composition according to claim 26.

32. A method for immunologically tolerizing a subject to one or more HIV gp120 epitopes comprising introducing into said subject an effective amount of transformed cells according to claim 17, thereby tolerizing said subject.

33. A method for immunologically tolerizing a subject to one or more HIV gp120 epitopes comprising introducing into said subject an effective amount of transformed cells according to claim 18, thereby tolerizing said subject.

34. A method for immunologically tolerizing a subject to an HIV gp120 epitope comprising introducing into said subject an effective amount of transformed cells according to any of claims 19-22, thereby tolerizing said subject.

35. A method of (i) inducing and (ii) maintaining immunological tolerance to an epitope or epitopes of HIV gp120 protein in a subject, comprising:

(a) administering to said subject an effective amount of a pharmaceutical composition according to claim 23 to induce said tolerance to said epitope or epitopes; and

(b) administering to said subject an effective amount of transformed hemopoietic or lymphoid cells according to claim 17 to maintain said tolerance to said epitope or epitopes,

thereby inducing and maintaining said tolerance.

36. A method for identifying whether a candidate HIV gp120 epitope or epitopes are tolerogenic in a first subject when presented to said subject in a fusion Ig molecule, comprising the steps of:

(a) stably transforming a population of hematopoietic or lymphoid cells of said subject with a vector according to claim 13 in which the gp120 epitope or epitopes in the fusion Ig encoded y said vector are said candidate epitope or epitopes;

(b) introducing said transformed cells into said subject; and

(c) determining whether said subject is tolerant to said candidate epitope or epitopes by measuring the reduction of an ongoing antibody response and comparing with a similar response in a second control subject who is (i) untreated or (ii) treated with control untransfected

cells or (iii) treated with cells transfected with a control Ig lacking said candidate epitopes;

such that, if said first subject is tolerant, said candidate epitope or epitopes in said fusion Ig is identified as being tolerogenic.

37. A method according to claim 36, wherein said first and second subjects are humans.

```
?e?lWVTvYyGVPVWkeatTTLFcASdakaydtEvHNvWathAcvPTdPn      50
TDKK    I        RDTA     G   ERRTSAK I   I DKQ RI   N S
KGQS                N            SHKA A                 D
AAEF                I              SR K
VNNT                S              E  M
ETDV                               H  R
Q E                                G  G
  R
  V

Pqevvl?nVTEnFnmWkNnmVeQMhEdiisLWdqsLKPCVkltPlCVtLn    100
 RKIEMGS    D DI T DI D   Q NVVN   NEA      TIA I   S K
 H LK V     H  A E S  K   N G S    G R      Q P       H
 K MP Q     K    D P  Q   Y                            I
    L E     S                                        D
    I K     Y                                        E
    A I     E
    T T
    F
    N
    R
    H

ctd?????ntn???????m e?geikncsfnittsirdkvqkeyalfykld    150
 KHLKNATKSSSSSWEEKE IKEDMRTR   YVSKILKGEIKRTNSFLRNHN
 INVIADPINIGNDGRGMV GRSGLQ     KTNSNRGNRMRTAS TYHRTY
 HAYLDVGRAATTNEWRAN DGAVVT     QA AGVST KTEDH H SSF
 N WLKTS DRAKWRMTTK KIP        EG  AKIS TEIQF Y NEP
 A FG NN  TNGVSIANI MER            DM E L  KT Q DTS
 D AT PR   VLKMTLGR HN             RS   E  G     GI
 F EV IA   IATNSISD AP             E    I  N
   KR SL   PEEAGSIP T              M    R  V
   N  C    CNIVKMLS P              T    G
   D        DRGK VEA R                   F
   C          YT  P                      Y
               D  R                      A
```

FIG. 1A

```
vvpid????? yrlisCntsvitQacPkvsFepipihyCaPaGfailkCnd      200
IISTNKDTTN FTMTH DRTTTK TY RIT Q VLMYF T T YVLIQ RN
LAQVGNKKSS  K MN IN IL         G    Q  V G LGM N KG
 EAMERNSYK  I RK HS VV         D       P    T  R  E
 MKLADAPRD  M VD K  L                   G
 L  KGGYKT  P
    QHSDNR  A
    H TNGY
      E D
        R

kkFnGtgpctnvStvQCthgIrPvvstq llNGSlaeeevvirseNftdN      250
TT S KEQWKKI AI  A R K IAVSH TS   QSGGDIIVKAD LAN
RR K S T ND            A  NS       KKK ML YA SSG
PN T N S S             T            AG     V  ME
EM S R K R                                  K
NQ H   F E                                  S
QE D   Q                                    Q
       L
       E

aktiivqlnesveinCtRpnnntrksihi??gpgrafyt tg?igdirqah      300
IRNMTIHPKQAIVMD I VGIYRAGGVNMQRAFKSTLHA IERAENTSKPY
T ILL  VRTT D K   ASKTAIQRLGLGHHLRKVIVY RRKV TMKE
V V    KDAP K T   LYHKKKRTMYT   IW GKWFV KKET YL R
H S      KY Q S    HYSVSNH TS    G QRYRR ADQL I  L
         V  A       THIT K SK    A  SV   SNDM
         D  T         SQ   PV    S  Q    N NK
            N              R                 H
            L              A                 G
                                             I
                                             P
```

FIG. 1B

```
CnisrakWnntLkqiv?kLreqf??nktivfnqssgGd peivmhsfnCgG    350
 DVNGTQ KAA QKVATT KNRLGNKNNKISKKPAE E LVVETLTLI R
 TLDISI TD  NRTFDR QKKYERER  AVTHAL    Q GMLYN T W
 A  KKR HK  MNLTG  GVP VGAT  K DRHT    R  TFNM   A
 Y  TVA ES  GLRGK   AL RV Y  R QP         AQPI
 V  EEN QE  E  EN   DH DE I  N RS          R
    SGD DV  R  DI   GE KH    M FE
    E S AR  W   E       K    T S
      E S   S   S       Q    S
      T Y   H   R       D
                M
                A

eFfyCnttqLfnstw?n?t??????n?t?????i tLpCrikqiinmwQev    400
K LF KSAK YSNA LYHNWSTEEGSNSEGNDTL I Q KTREFVDRR K
G    DASP  DTK PENSGGPKGSDDGDESEPT K L   S VLRLC V
     S RH    IRFSSLADSRKLKGESRENN  V G       YV  G
        G    E SMDVEIKGSTTIINKRRL    S        S  Q
        N    S R V DTWNIE TNQNKSI              K  R
             N K T SNGTTR VMK TGD
               W E YFNQWN LD  GVS
               G G REISLK S   YAE
               A F V DYMV A   AI
               E I   VARW Y   DL
               T       GDQ R   VK
               V         A  C
               D            E
               N            K
                            I

GkamyapPi?gqircssnItGllLtrDgGn???? ?eiFRpgGGdMrdNwR    450
 RPLSDL AGRNVSSLLK   MI V    KTENN DAV  LA  N KE R
 Q   V   S YLK T H      L    TSTMR N T   V  A  G
 P       K T N I             DAKIS G       E
         R H T V             SNNDK T
         E L Q W             EEGTT S
         D K I                RPEE R
         Q P                  ISS  E
         T                    GHG  A
         N                    DQ   K
         A                         Q
                                   H

seLYkYKvvkieplGvAptkakrRvvqrekR                        481
NK  R  IITVKLI I SSRPRK TMRGKR
 R       E V   L  NT NS   K Q
 V       R         Q       E
         Q
```

FIG. 1C

signal peptide / gp120

```
CONSENSUS-A   Mrvmgiq?nyq?l.wr??....??W.gtmilg??iIc.na??e.?lWVtVyYGVPVWkdaeTTLfcAS   49
CONSENSUS-B   ??---k--rk---h-?----????---.---l--mlm--.s----.----------------e-t-------   53
CONSENSUS-C   ---r--?r-w-qw.-i.....-.-ILGFwmlm--.-v--g.n----------------e-k-------   53
CONSENSUS-D   ---r?-er---h-.----????---.---L--mLM--.sv.a??----------------E-t-------   52
CONSENSUS-E   ---Ket-m-wpn-.-k--.....---.--l---lv---?s-.Sd.N----------------r--d-----   55
CONSENSUS-F   -?-R-M-R-W-H-.GK.....-.-LLF--iL---.--.--.n----------------e-T-------   53
CONSENSUS-G   -?-k---r-W-H-.-k.....-.--L---LV----.s-.sn.n----------------E--D-------   54
CONSENSUS-O   -t-tMKaM?KrNr.Kl.....-?lylamALi-P-.LS.-??Q-YA---s-----E--?Pv-----   51
CONSENSUS-U   -?-?E?-R-??-?.-?.....-.???-----?--.?-.-??-?----------------------   36
CONSENSUS-CPZ -?????-???-?.??--?.....?????-?--???.?T.---.--??---?------??-?P?---?   19

CONSENSUS-A   dAkAydtE?HNVW?aTHaCVPTDPnPqEi?le.NVTE?FnmwkNnMVeQmheDiiSLWD.qSLkPCvkLt   113
CONSENSUS-B   ----------v----.----------------vv-??----n----------.----------.----------   119
CONSENSUS-C   -----e?-v-----.----------------mv--.----n------d--d----------.----------   119
CONSENSUS-D   ---s-k?-a--i-.----------------.----N----------.----------.----------.----------   117
CONSENSUS-E   ----He--v-----.----------.----n----------g--v-----?----------.----------   121
CONSENSUS-F   ---S-Ek-v-----.----------Vv--.----n-d----------T----------.----------.----------   120
CONSENSUS-G   -----s--s-----.----------.----n----------.E----------.----------.----------   120
CONSENSUS-O   --NLTS--q--I-..-sQ-------?-?-yp-?.---d---I---Y--d----------.----------qM-   114
CONSENSUS-U   ---?-??-------.----------?--?---?-.----------?----------.?----------.----------   91
CONSENSUS-CPZ ?-???S-----?-.--??---?--?-??V--?.-???---??--?--??-?--???-??.--?------   56
```

FIG. 2A

```
CONSENSUS-A   PLCVTL?C.???????????n?t??????????n?t???????n?????...????????.........m   126
CONSENSUS-B   ------n-.td---------?-?------------------------???---------??...????-   133
CONSENSUS-C   ------n-.-----.....--?---.....------------t---?...------------.......??   132
CONSENSUS-D   ------n-.t----------?--------...-----------t---?...------??????????   131
CONSENSUS-E   ------n-.tna-----...-l---.---nv--i-nvsniig-it..................?????   150
CONSENSUS-F   ------n-?t-a--......-a---------t-?-?-q--......................?tLkE   139
CONSENSUS-G   ------n-.t-----.....-V--t--......?-?........NcT--?en--nNstv-.......???   143
CONSENSUS-O   F---QMn-.td-------.......------l--...----.................................   129
CONSENSUS-U   ------n-.t---........-----..........---e-.................-----..........   105
CONSENSUS-CPZ -?-???--.---------..............?-?-----...........-------P????..???    60

CONSENSUS-A   ?..?e.....ikNCsfNmTtelrdkkqkvysLfYrlDvVqi????????n??????....n?????????   160
CONSENSUS-B   e??g-?????-------i--si---v--e-a---k----p-d-------?------????----------   169
CONSENSUS-C   -?.--.....-------a---?--------A------i-pl-----.-----------....--s--.----   166
CONSENSUS-D   -?.-g.....m------i--?v----kq-ha---k---------------------.....--t--.----   165
CONSENSUS-E   -..d-.....Vr-----------------hA---k--i---------.---s----?...?--..----   185
CONSENSUS-F   eP.ga.....-Q---------v---Q?--Ha------I-p-s---...--ns---....??--.------   177
CONSENSUS-G   -..e-.....m------i---i----ktE-A---k----p-n---...-?ss-.......----..---sd   182
CONSENSUS-O   ...n-??...m-?-?--V--V-k---E-KQA---Vs-L?k?N-ts--..--T---..............m   164
CONSENSUS-U   -??-?.....-------i---?----kt?-a---k----P-n---....-n---......?--.......-   137
CONSENSUS-CPZ -??-?.....???-?--??-???---??????--??-????--.......-.........--T.....--    73

CONSENSUS-A   YrLInCntSaitqACPKvsFEPIPIhYCaPAgfAILkC?dk?FnGtGpckNvStVQCthg?ikPvvsTQL   227
CONSENSUS-B   ----s----v---------------------------n--k-------t----------.-r-------   238
CONSENSUS-C   ------------------d-----------y-----nn-t-------h----------.---------   235
CONSENSUS-D   -----------------t------------------n--k-----------------.-r-------   234
CONSENSUS-E   ---------V-K-----i--D-------t---y-----N--n-----------S------.---------   254
CONSENSUS-F   ----?----T---------Wd-----------Y-----N--k-----------------.---------   245
CONSENSUS-G   -------v-T-K------n-d-----------------r--n-----------------.---------   251
CONSENSUS-O   -?-t---STt-?-------------------y--F--N?T------l-?-itV-T----.---T-----   228
CONSENSUS-U   ---------?-k-----------------------n--K-------------------.---------   205
CONSENSUS-CPZ -????---T?---?-?-??-------------?---?---?D-?-?-?-?---?-H----.-?-?-?-?-   120
```

FIG. 2B

```
                          <- V3 neutralization loop
CONSENSUS-A   LLnGSLAe???v?irSenitnNaktiiVql??pV?InCtRP.nnntr.ks???vri???gpGq??afya.   279
CONSENSUS-B   --------e.e-v-----f-d---------nes-e------?-----.----.ih-------r.---t.   296
CONSENSUS-C   ---------.eii-----l---v-----h-n-s-e-v---.-----.--...i--..-----.t---.   291
CONSENSUS-D   --------E.EiI-----l-----?-----nes--------?y?---.qr..-tp-...-?--..-l-t?   288
CONSENSUS-E   --------e.eIi-----L---------h-NKs-e------.s----.t-...it-..------.v--r.   312
CONSENSUS-F   --------e.dii---q--sd-------h-Nes-q-----.-----.--...I?-..---r.-----.   302
CONSENSUS-G   --------e.eI------?-d---v-----nksie-?---.-----.--...l?f..-----.-----.   305
CONSENSUS-H           -.------?---D-T-N-----K----------.-----.?---.I?-...-?--..?-?-.    39
CONSENSUS-O   I---T-Skg.kIr-Mgk--?dsg-N---T-N-?i-mt-e--.g-?-v.Qe...i?-....--.m..-W-S.   279
CONSENSUS-U   --------E.E-i---?---d---------net-k------?-----?--...?--..-----.v---.   261
CONSENSUS-CPZ -?--????---?-?????K?????V?????-E??-??-?--.G-?-?.??...?Q-....---M..T--N.   142

            V3 neutralization loop ->                                       CD4
CONSENSUS-A   tgdi.....iG.dirqAhCnvsr?eWn?tlq?V....a?qLr?..?f???nkt....??iiF?n.ssGGD   320
CONSENSUS-B   --?-???..--.---------i--ak--n--kqi....v-k--e??q-.-------....---v-nq?-----   342
CONSENSUS-C   ----.....--.---------I-?-k--e-------....?kK-ae..h-p----------.....-k--?.-----   334
CONSENSUS-D   -?r????..?-?---------i-?a?--k---q-....--k-gd?.ll.--------.....t---kp.-----   331
CONSENSUS-E   ----.....--.---k-y-EINgTk--e?-kq-....tek-ke..H-..n-----------.....---qP?p----   360
CONSENSUS-F   --?-.....--.---k------gtq-----e--....?a?-ks..h--.--?-.....--k-ns.-----   344
CONSENSUS-G   ----.....--.-------------?-?em--n-....?-?-?-..i------?...?---t-ns.-a---   344
CONSENSUS-H   ?-?-.....--.---?-?--I??-?-?-?-?--....?---?-..H?.--??.....--?--P.-----    65
CONSENSUS-O   M-.l???n?k???s-?-Y-?YnaTd-?ka-kqt....-eRYLe..Lv...-?-????vtm?-n?s-?---   321
CONSENSUS-U   ----.....--.---------i--t?--n---q-....-----k..y-..n-?-.....--?-ns?-----   306
CONSENSUS-CPZ ?E??.....?-.-T-?-?-??N?T?-?-???-??????-???-..-?...-?-...?A-???-?.???--   157
```

FIG. 2C

```
CONSENSUS-A   lEitthsFnCggef?FYCnts?lF.nstW???????....n?t.??????.??n?t????..???sndtI   355
CONSENSUS-B   p--vm---------.-----tq--.-----------????---?------?---------??---????-   374
CONSENSUS-C   ----------r---.---------.---y----p-...?-g-?------?-.--?-...??---????-   366
CONSENSUS-D   p-------------.---------.-----------????--??---.--?..----.--??---????-   361
CONSENSUS-E   ----m-h---r---.-----t---.-n-cig-........-e-m....-gc..-g-.........????-   398
CONSENSUS-F   ----m-----r---.---------.-?-?-----......---?--......?-?---...---???--   372
CONSENSUS-G   ----------r---.------g--?-?s?---........-n??------?.---?---....--????-   373
CONSENSUS-H   ?--??-?---?---.-----?K--.--------.......--??--.N--?..-G-...........??-    92
CONSENSUS-O   ?-v-hlh---H---.-------m-.-y-Fsc-----????---?------?-----n--......?-g-?   356
CONSENSUS-U   ?-----t-------.---------.--?---.........---?d-...-?.------.....---????-   336
CONSENSUS-CPZ P-V??-?---?---.---???-?-.????--........?I-.....................-G??   175


CONSENSUS-A   t..lq.CrI.kqIvnm.wQrvgq.AmYapPIq.g?irc?sNITGllLTRDGg??....nns??...????   401
CONSENSUS-B   -??-p.---.---i--.--e--k.-------?.-q---s--------------????-??--?.?----   419
CONSENSUS-C   -..-p.---.---i--.--e--r?-------?.-n-t-k--------?------???.-?t--???----   411
CONSENSUS-D   -..-p.---.---i--?--?--k.-------e.---?-s---------------?...-?--...?----   405
CONSENSUS-E   -..-P.-k-?---i--.--ga--.-------s.-r-n-V-----I-------a-?...--t-........   447
CONSENSUS-F   -..-p?---.------.--e--r.----a--a.-n-t-n---------------??..-?t--?.....-   419
CONSENSUS-G   -..-p.---.----r-.------.-------A.-n-t---------------n-....--t-.......-   421
CONSENSUS-H   ?..-?.---.---??-.--?--.-------?.-K-T--------I--S-HV......-?-AE.......   134
CONSENSUS-O   ?..iP.--L.r-v-Rs.-m-G-S.gl-----?.-NLt-------MI-Ql-?.pW....-s---......--   401
CONSENSUS-U   -..-P.-?-.----r-.--g---.-?-----a.-----------?------n-?...-?t........-   379
CONSENSUS-CPZ I..??.---.?---??.-??-??.GI-??-?R.-N??---?---??-?????--....-??.........   198


                                                               gp120 / gp41
CONSENSUS-A   ?netFrPgGgdmrdNWrsELYkYKvVkiePlGvaPtr.akrRVV....eREKRA??vg.lGavflgflGa   462
CONSENSUS-B   -t-i-------------------------------k.-------....q-----.--?i--m-------   480
CONSENSUS-C   -?----------------------e-k-------?.-------??..------.---?i---------   470
CONSENSUS-D   ------------------------r---------?.------...-------..I-.---m-------   465
CONSENSUS-E   ----------NiK-------------Q-----i---.------...-------.--.I--Mif-----   508
CONSENSUS-F   -?--------n-k-------------e-----------.---q--....k---------.?--l-------   478
CONSENSUS-G   ---------------------------k---------.-R-----....G-----.--.?----------   481
CONSENSUS-H   -?V----------------------??----?---?.-?-----....?------.--.-?---------   187
CONSENSUS-O   --?-l--?----k-I--T--f-----rvK-FS----ki-RP?Igt?t?H-----.--.--ML---v-S-   462
CONSENSUS-U   -?-----------------------------------.??----....?-----.---.M--?-------   435
CONSENSUS-CPZ -?????-??????-?--?--??-?--?-?--S----???R???????.?-Q--?.-?-.?-??---?-?-   227
```

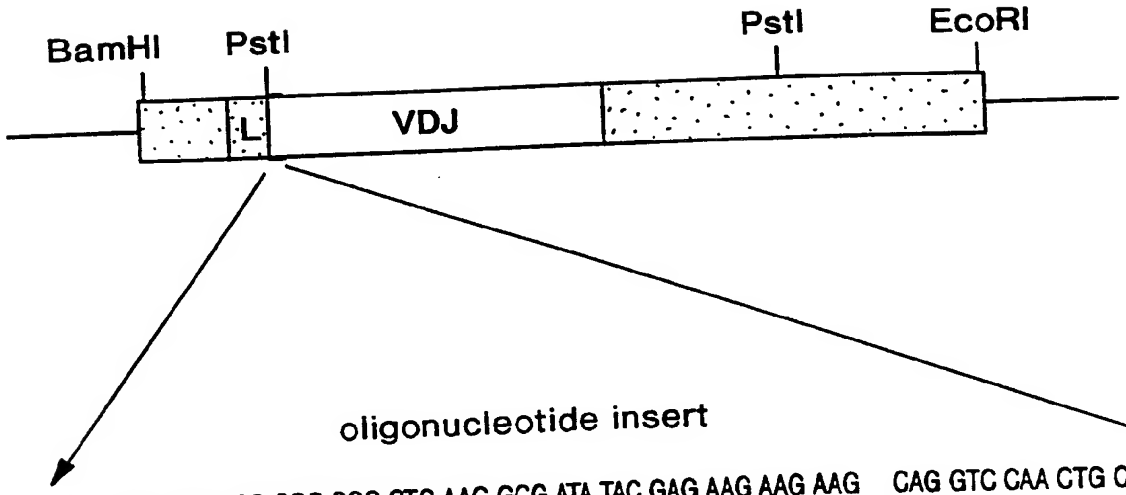
FIG. 2D

```
                ?? ir  n          ????????
CON-B[2]        mrvkgkyqhl?wrw???↑Gtm llGmlmicsa//?e?lWvTvYy GVPVWkeatT TLFcASdaka
BH10[3]         MRVKEKYQHL WRWGWRWGTM LLGMLMICSA//TEKLWVTVYY GVPVWKEATT TLFCASDAKA  60
BRU[4]          MRVKEKYQHL WRWGWKWGTM LLGILMICSA//TEKLWVTVYY GVPVWKEATT TLFCASDAKA  60
SF2[5]          MKVKGTRRNY QHL.WRWGTL LLGMLMICSA//TEKLWVTVYY GVPVWKEATT TLFCASDARA  59
MN[6]           MRVKGIRRNY QHW.WGWGTM LLGLLMICSA//TEKLWVTVYY GVPVWKEATT TLFCASDAKA  59
92US712.4[7]    VTEIRKNC QHW WRWGIM LLGMLMTCNN//AEESWVTVYY GVPVWKEATT TLFCASDAKA  57

                                    ??
CON-B           ydtEvHNvWa thAcvPTdPn PqevvlↆnVT EnEnmWkNnm VeQMhEdiis LWdqslKPCV
BH10            YDTEVHNVWA THACVPTDPN PQEVVLVNVT ENFNMWKNDM VEQMHEDIIS LWDQSLKPCV  120
BRU             YDTEVHNVWA THACVPTDPN PQEVVLVNVT ENFNMWKNDM VEQMHEDIIS LWDQSLKPCV  120
SF2             YDTEVHNVWA THACVPTDPN PQEVVLGNVT ENFNMWKNNM VEQMQEDIIS LWDQSLKPCV  119
MN              YDTEVHNVWA TQACVPTDPN PQEVELVNVT ENFNMWKNNM VEQMHEDIIS LWDQSLKPCV  119
92US712.4       YDTEVHNIWA THACVPTDPN PQEVVLENVT ENFNMWKNNM VEQMHEDIIS LWDQSLKPCV  117

                ???????????????? ???????????? ????????? ?????
CON-B           kltPlCVtLn ctdↆↆn?tↆↆ n?????????ↆ??meↆgeik ncsfnittsi rdkyqkeyal
BH10            KLTPLCVSLK CTDLKNDTNT NSS...S..G RMIMEKGEIK NCSFNISTSI RGKVQKEYAF  175
BRU             KLTPLCVSLK CTDLGNATNT NSSNTNSSSG EMMMEKGEIK NCSFNISTSI RGKVQKEYAF  180
SF2             KLTPLCVTLN CTDLGKATNT NSSNWK.... ..EEIKGEIK NCSFNITTSI RDKIQKENAL  173
MN              KLTPLCVTLN CTDLRNTTNT NNSTANNNSNSEGTIKGGEMK NCSFNITTSI RDKMQKEYAL  180
92US712.4       KLTPLCVTLN CTDLSNATNT NATTTTNSSA GMMMDRGEIK NCSFNVTASI RDKMQREYAL  177

                ??????????????? ??????
CON-B           fyklduvpidↆn???↑  yrlis cntsvitQac PkvsFepipi hyCapagfai lkCndkkFnG
BH10            FYKLDIIPID NDTTS   YTLTS CNTSVITQAC PKVSFEPIPI HYCAPAGFAI LKCNNKTFNG  235
BRU             FYKLDIIPID NDTTS   YTLTS CNTSVITQAC PKVSFEPIPI HYCAPAGFAI LKCNNKTFNG  240
SF2             FRNLDVVPIDNASTTTNYTN YRLIH CNRSVITQAC PKVSFEPIPI HYCTPAGFAI LKCNNKTFNG  238
MN              LYKLDIVSID NDSTS   YRLIS CNTSVITQAC PKIS.EPIPI HYCAPAGFAI LKCNDKKFSG  239
92US712.4       FYKLDVIQID N  TS   YRLIS CNTSVITQAC PKVSFEPIPI HYCAPAGFAI LKCNDKKFNG  235
```

FIG. 3A

```
CON-B       tgpctnvStv QCthgIrpvv stqlllNGSl aeeevvirse nftdnaktii vqlnesvein
BH10        TGPCTNVSTV QCTHGIRPVV STQLLLNGSL AEEEVVIRSA NFTDNAKTII VQLNQSVEIN   295
BRU         TGPCTNVSTV QCTHGIRPVV STQLLLNGSL AEEEVVIRSA NFTDNAKTII VQLNQSVEIN   300
SF2         KGPCTNVSTV QCTHGIRPIV STQLLLNGSL AEEEVVIRSD NFTNNAKTII VQLNESVAIN   298
MN          KGSCKNVSTV QCTHGIRPVV STQLLLNGSL AEEEVVIRSE NFTDNAKTII VHLNESVQIN   299
92US712.4   TGPCKNVSTV QCTHGIRPVV SSQLLLNGSL AEEGVVIRSA NLSDNAKIII VQLNESVEMN   295

             ?        ??    ?                ? i  qdi                ??
CON-B       CtRpnnntrk sihi??gpgr afyttg?i?? irqahCnisr akWnntLkqi v?kLreqf??
BH10        CTRPNNNTRK SIRIQRGPGR AFVTIGKIGN MRQAHCNISR AKWNNTLKQI DSKLREQFGN   355
BRU         CTRPNNNTRK SIRIQRGPGR AFVTIGKIGN MRQAHCNISR AKWNATLKQI ASKLREQFGN   360
SF2         CTRPNNNTRK SIYI..GPGR AFHTTGRIIGDIRKAHCNISR AQWNNTLEQI VKKLREQFGN   357
MN          CTRPNYNKRK RIHI..GPGR AFYTTKNIIGTIRQAHCNISR AKWNDTLRQI VSKLKEQF.K   357
92US712.4   CIRPNNNTRK SIHI..GPGR AFYTTGDIIGDIRKAHCNISR AKWNNTLKQI AIKLKEQFE    354

              ?v?                                 ?????????    ????  ???????? ??
CON-B       nkt?ifnqss gGdpeivmhs fnCggeffyC nttqLfnstw-?n?t??????-n?t??-??it-
BH10        NKTIIFKQSS GGDPEIVTHS FNCGGEFFYC NSTQLFNSTW FNSTWSTKGS NNTEGSDTIT   415
BRU         NKTIIFKQSS GGDPEIVTHS FNCGGEFFYC NSTQLFNSTW FNSTWSTEGS NNTEGSDTIT   420
SF2         NKTIVFNQSS GGDPEIVMHS FNCRGEFFYC NTTQLFNNTWRLNH   TEGTKGN....DTII   412
MN          NKTIVFNQSS GGDPEIVMHS FNCGGEFFYC NTSPLFNSTWNGNNTWNNTTGSNNN.....IT   414
92US712.4   NKTIVFNQSS GGDPEIMTLM FNCGGEFFYC NSTQLFNSTW .NSTQLVND. ..TGGN..IT   407

                                                         ????????
CON-B       LpCrikqiin mwQevgkamy apPi?gqirc ssnItGllLt rDgG???n????t eiFRpgGgdM
BH10        LPCRIKQIIN MWQEVGKAMY APPISGQIRC SSNITGLLLT RDGGNSNNES... EIFRPGGGDM   475
BRU         LPCRIKQFIN MWQEVGKAMY APPISGQIRC SSNITGLLLT RDGGNNNNGS... EIFRPGGGDM   480
SF2         LPCRIKQIIN MWQEVGKAMY APPIGGQISC SSNITGLLLT RDGG..TNVTNDT EVFRPGGGDM   472
MN          LQCKIKQIIN MWQEVGKAMY APPIEGQIRC SSNITGLLLT RDGGKDTD TNDT EIFRPGGGDM   475
92US712.4   LQCRIKQIIN MWQEVGKAMY APPIRGQIRC SSNITGLLLT RDGGINKSE NGT EIFRPGGGDM   469

CON-B       rdNwRseLYk YKvvkieplG vAptkakrRv vqrekR
BH10        RDNWRSELYK YKVVKIEPLG VAPTKAKRRV VQREKR                             511
BRU         RDNWRSELYK YKVVKIEPLG VAPTKAKRRV VQREKR                             516
SF2         RDNWRSELYK YKVIKIEPLG IAPTKAKRRV VQREKR                             508
MN          RDNWRSELYK YKVVTIEPLG VAPTKAKRRV VQREKR                             511
92US712.4   RENWRSELYK YKVVKIEPLG VAPTKAKRRV VQREKR                             505
```

FIG. 3B



cI lambda repressor 12-26 sequence

FIG. 4A

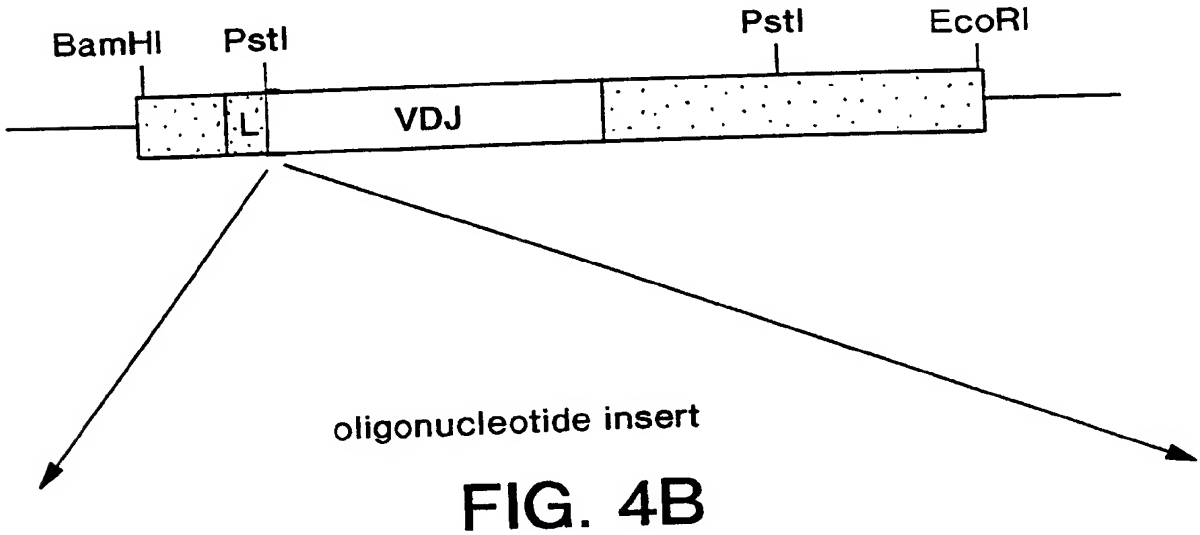


FIG. 4B

------FR1 sequence-------_____cI lambda repressor sequence_____----------FR1 repeat----------

CAG GTC CAA CTG CAG CTG GAG GAC GCG CGG CGG CTG AAG GCG ATA TAC GAG AAG AAG AAG CAG GTC CAA CTG CAG

L E D A R R L K A I Y E K K K

FIG. 5A

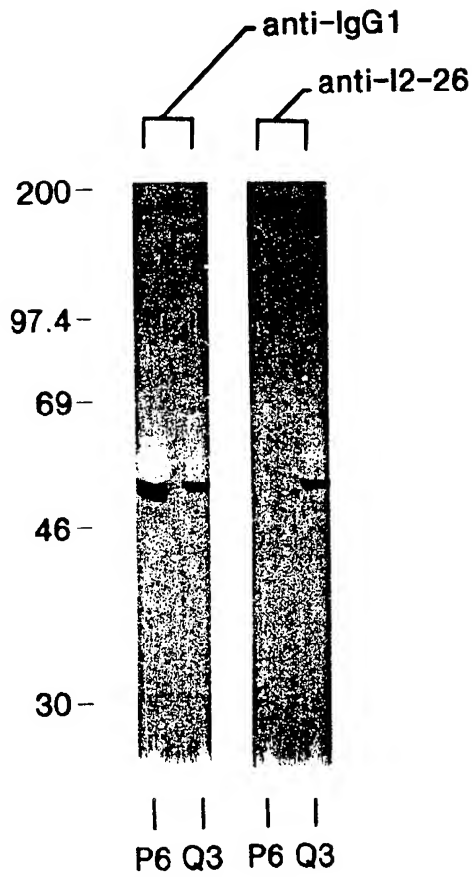


FIG. 5B

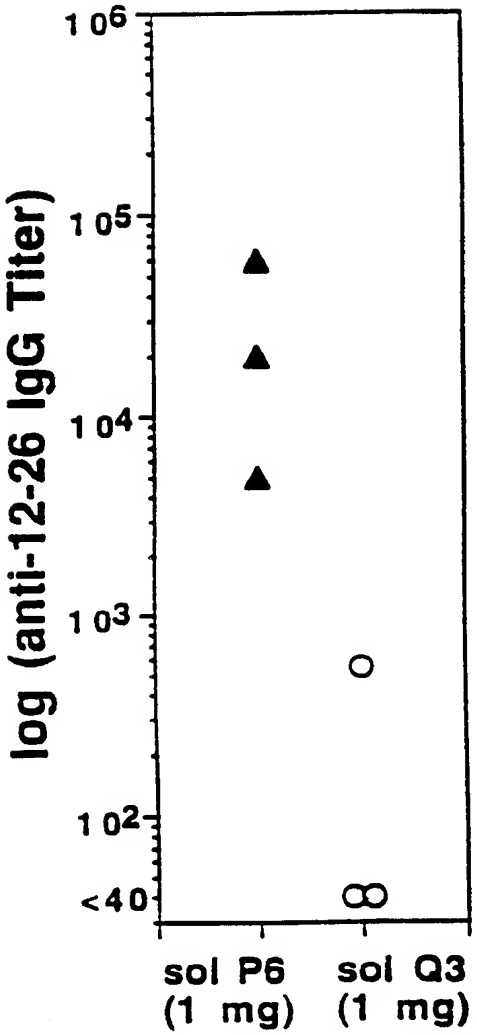


FIG. 6A

FIG. 6B

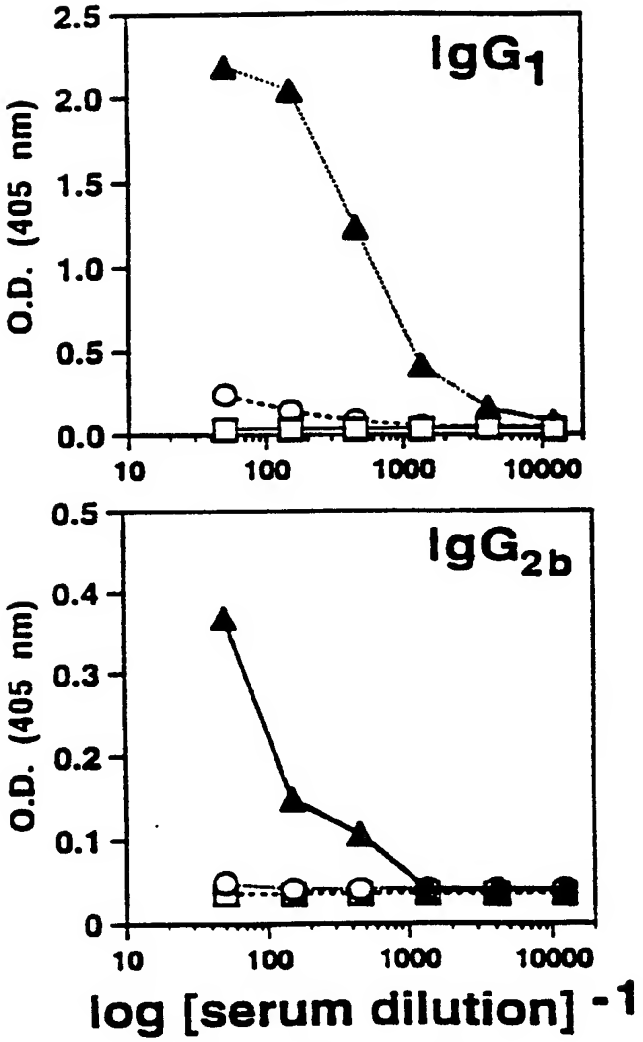


FIG. 6C

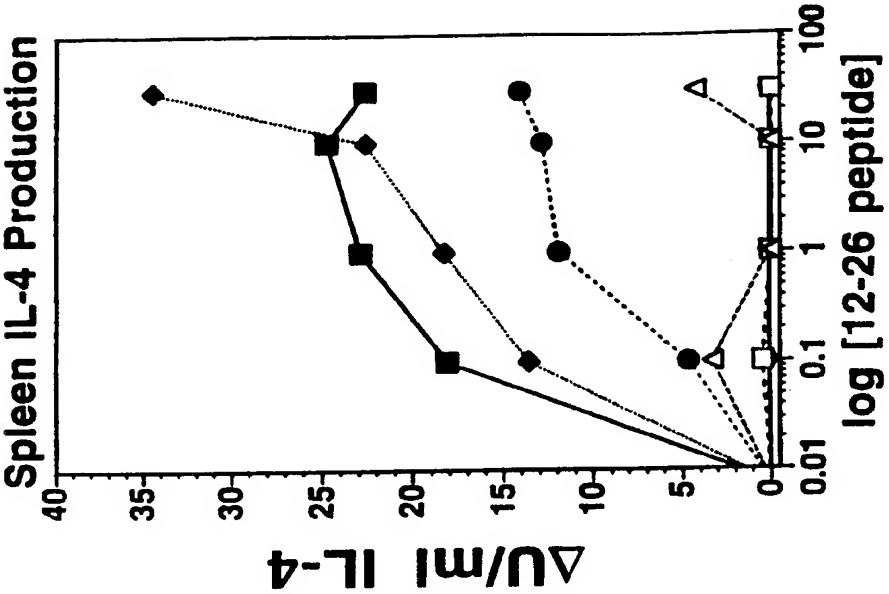

Spleen IL-4 Production
ΔU/ml IL-4
log [12-26 peptide]
0
5
10
15
20
25
30
35
40
0.01
0.1
1
10
100

FIG. 7B

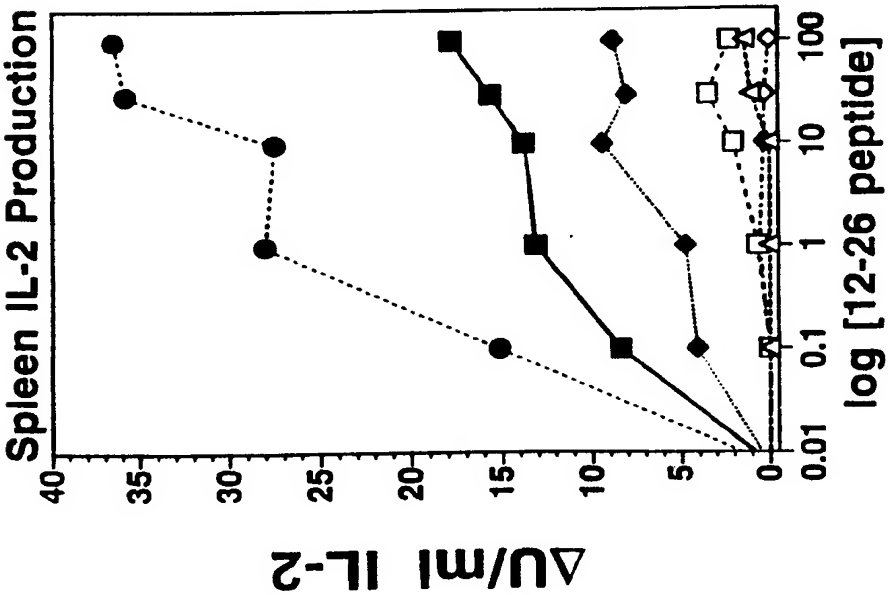

Spleen IL-2 Production
ΔU/ml IL-2
log [12-26 peptide]
0
5
10
15
20
25
30
35
40
0.01
0.1
1
10
100

FIG. 7A

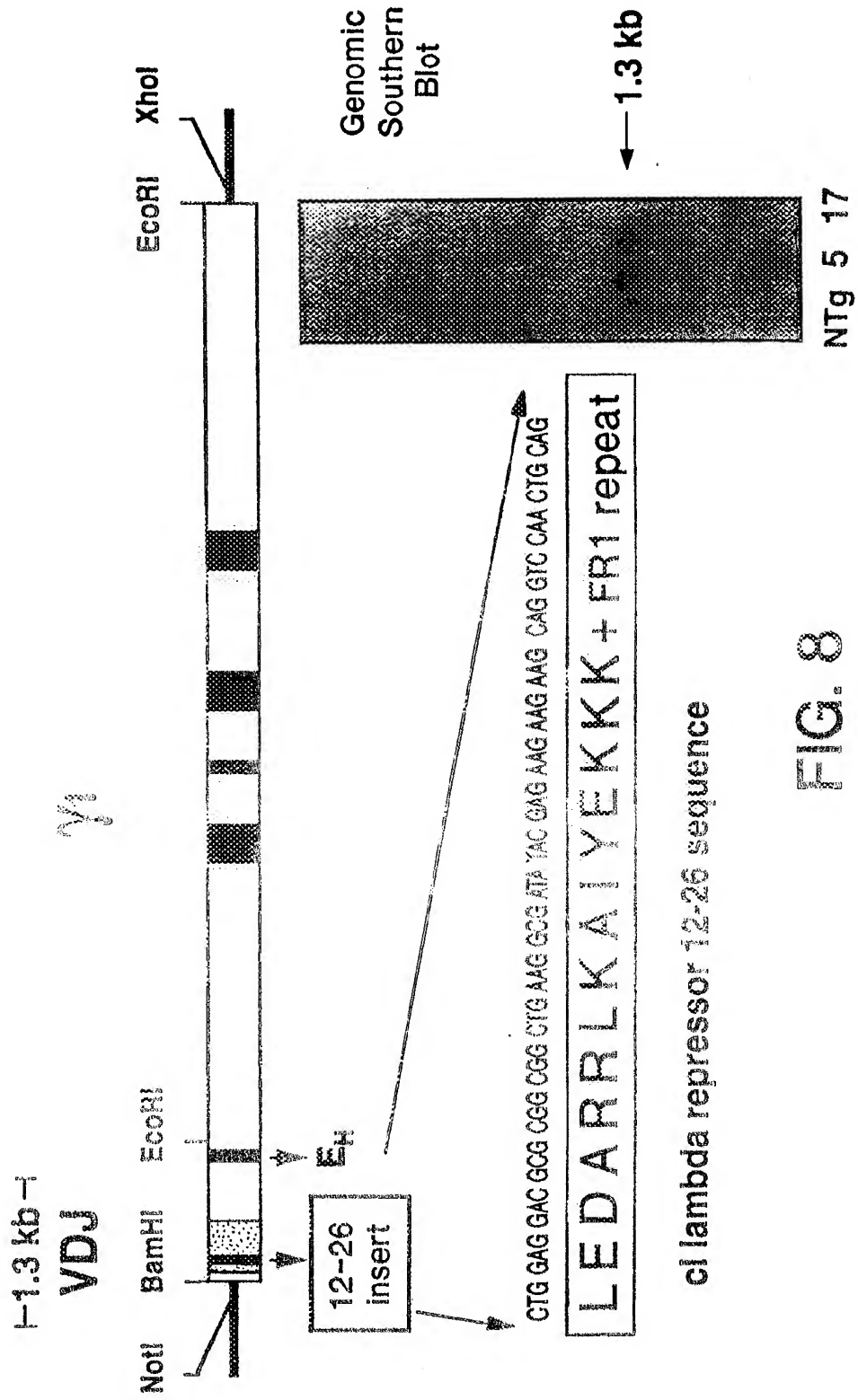

Genomic Southern Blot
1.3 kb
NTg 5 17
XhoI
EcoRI
1.3 kb
VDJ
BamHI
EcoRI
NotI
12-26 insert
CTG GAG GAC GCG CGG CGG CTG AAG GCG ATA TAC GAG AAG AAG AAG CAG GTC CAA CTG CAG
LEDARRLKAIYEKKK+FR1 repeat
cI lambda repressor 12-26 sequence

FIG. 8

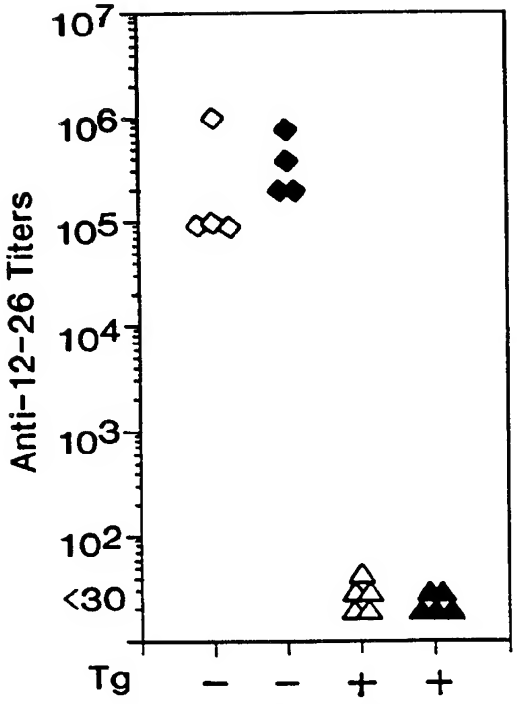


FIG. 9A

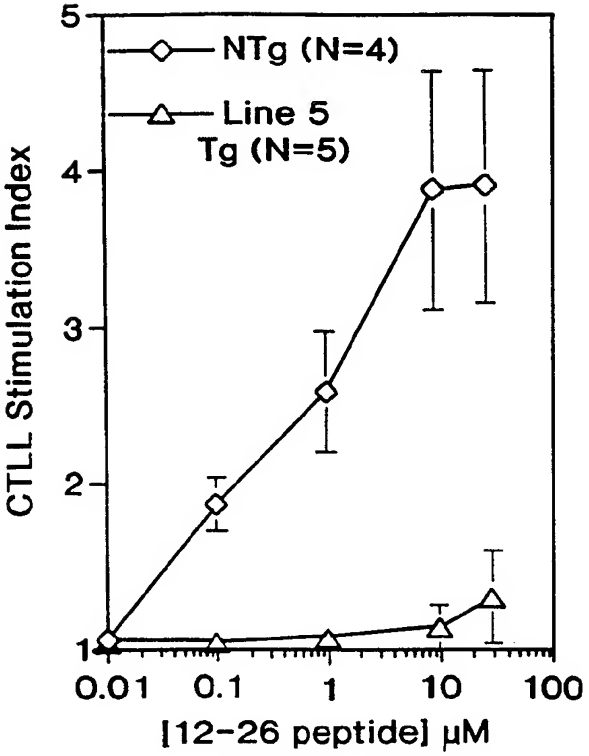


FIG. 9B

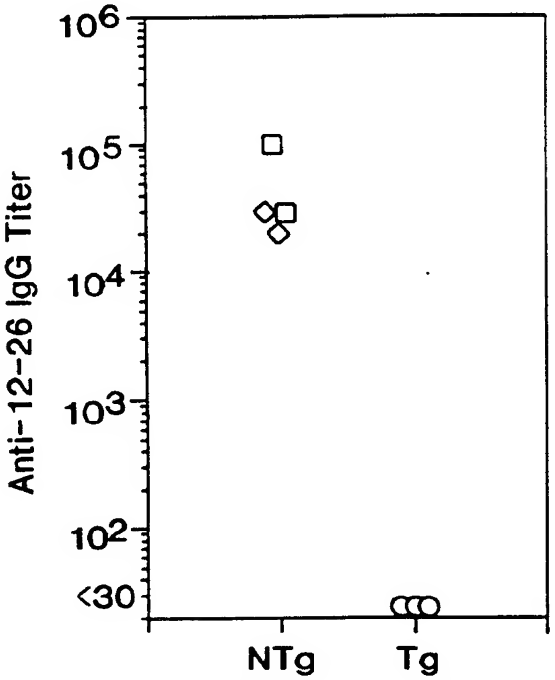


FIG. 10

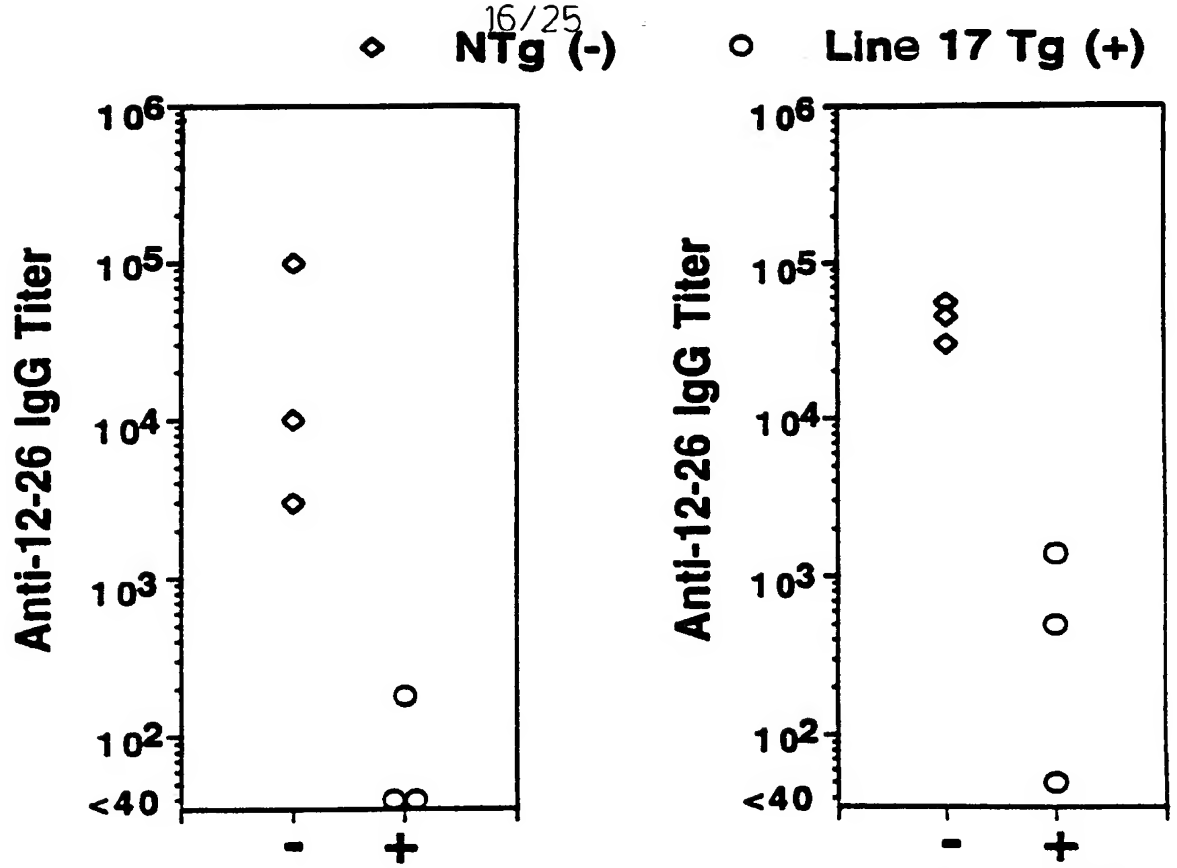


FIG. 11A

FIG. 11B

FIG. 11C

FIG. 11D

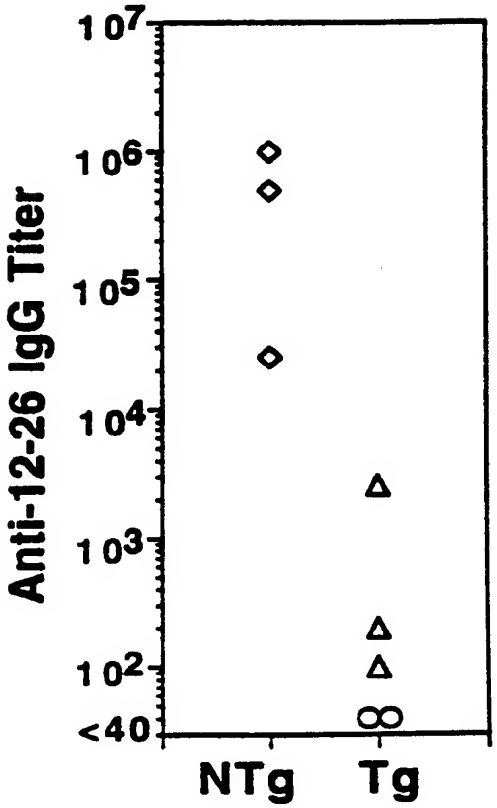


FIG. 12A

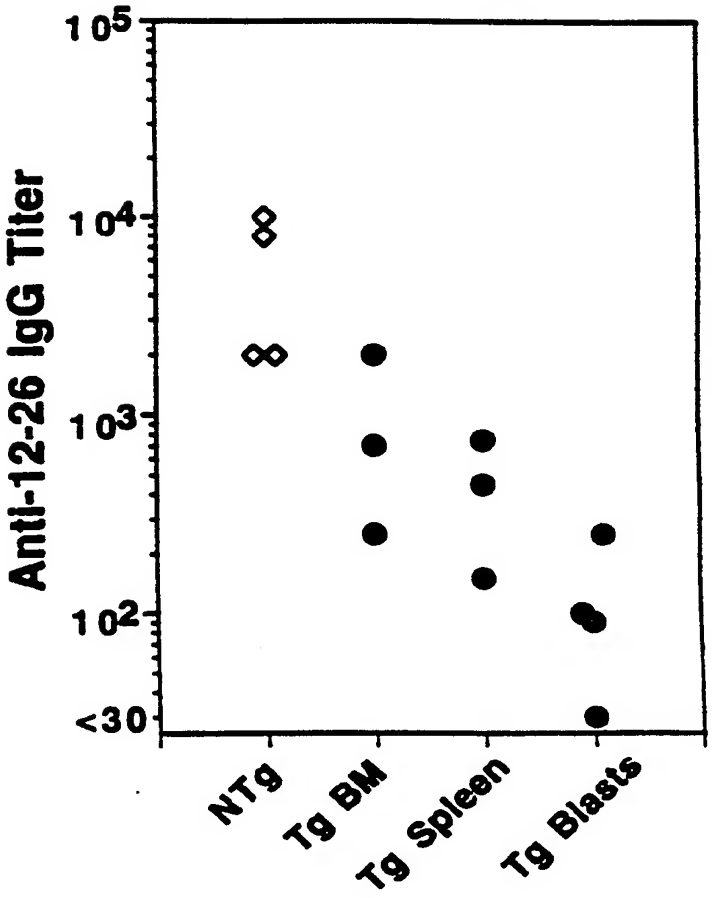


FIG. 12B

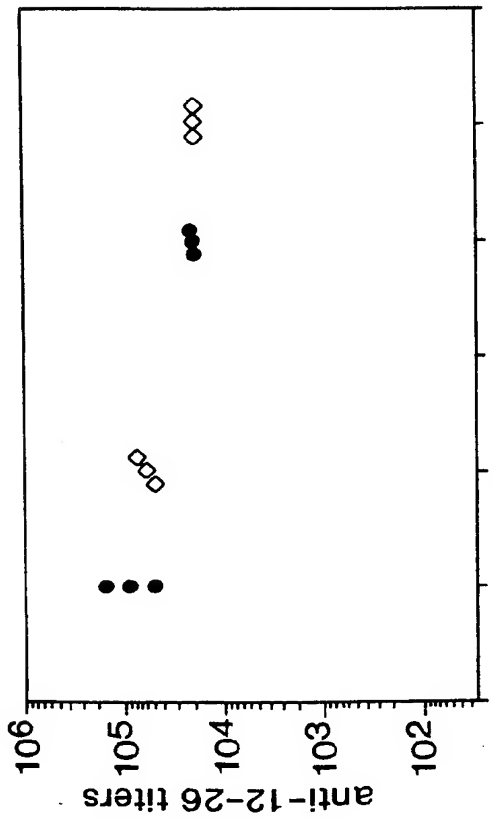


FIG. 13A

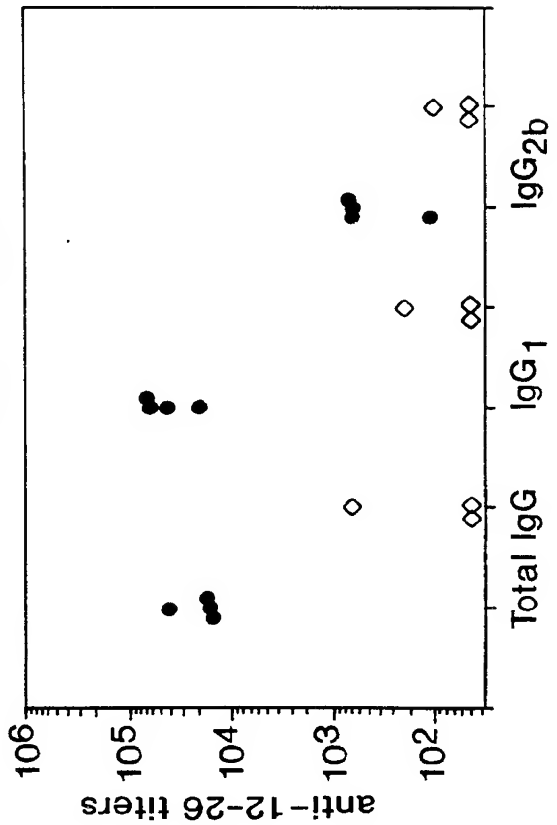


FIG. 13B

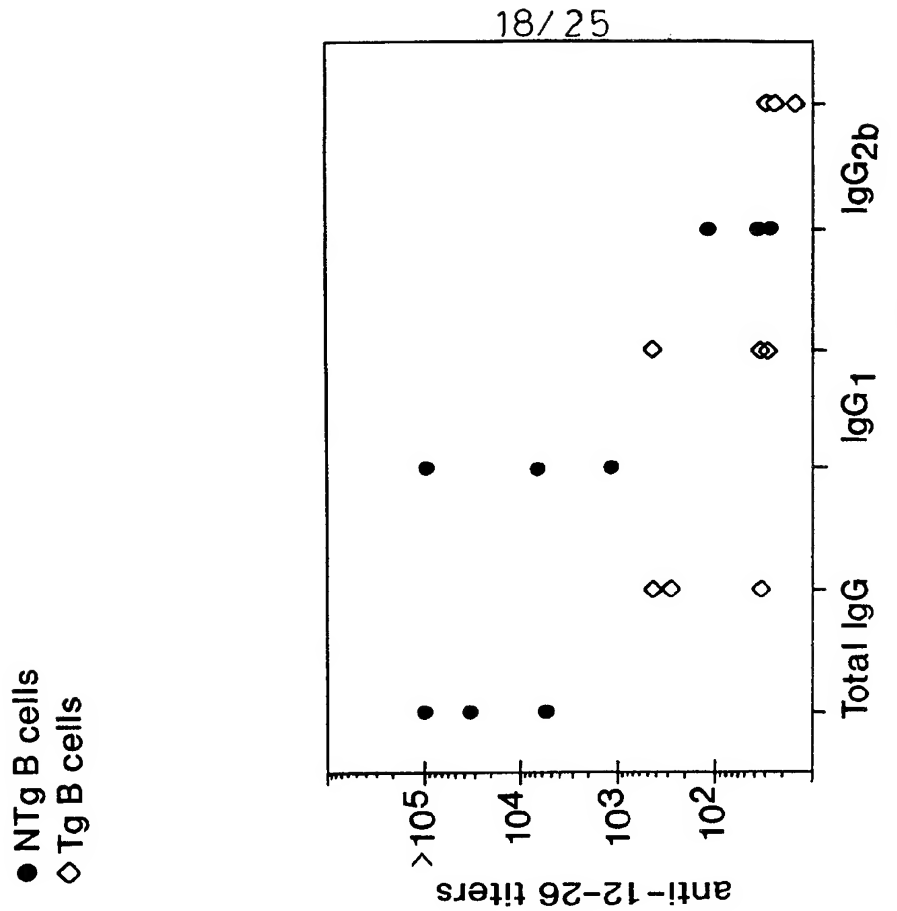


FIG. 13C

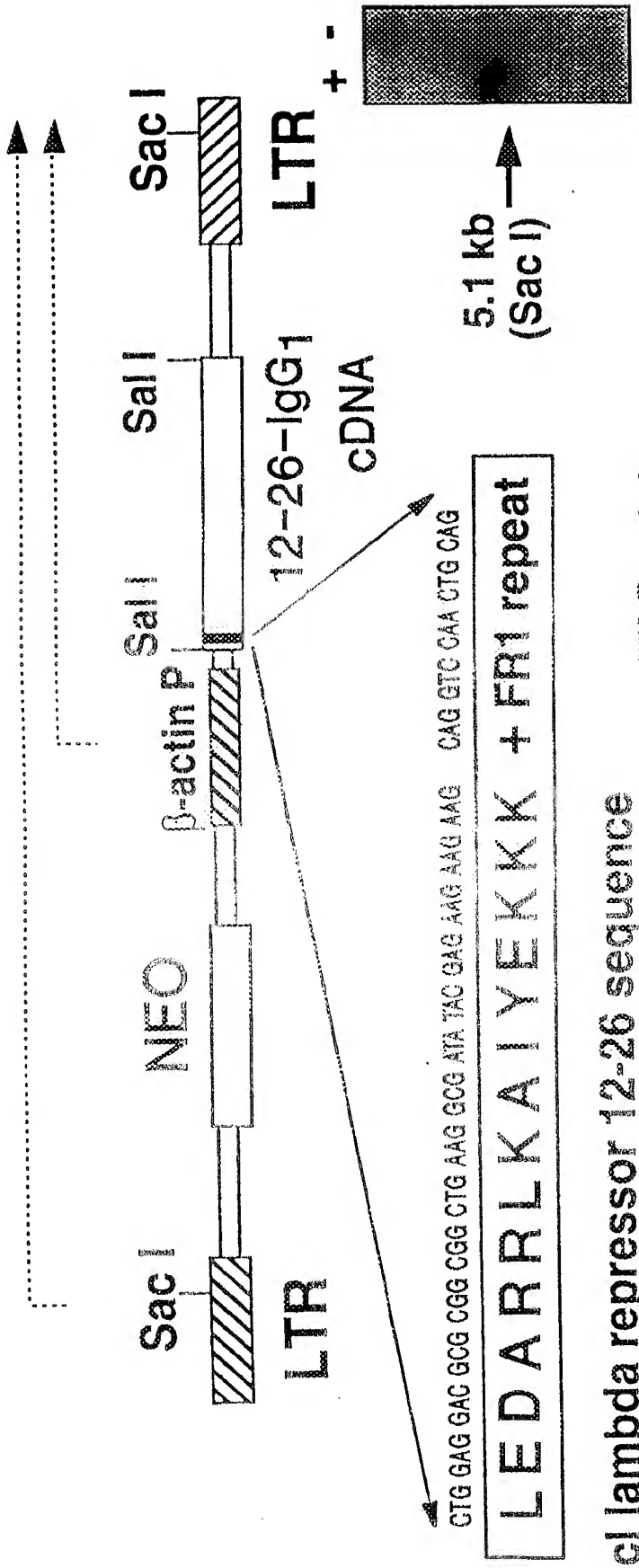

Sac I
Sal I
Sal I
β-actin P
NEO
LTR
Sac I
LTR
+ -
5.1 kb
(Sac I)
12-26-IgG1
cDNA
CTG GAG GAC GCG CGG CGG CTG AAG GCG ATA TAC GAG AAG AAG AAG CAG GTC CAA CTG CAG
L E D A R R L K A I Y E K K K + FR1 repeat
cI lambda repressor 12-26 sequence

FIG. 14

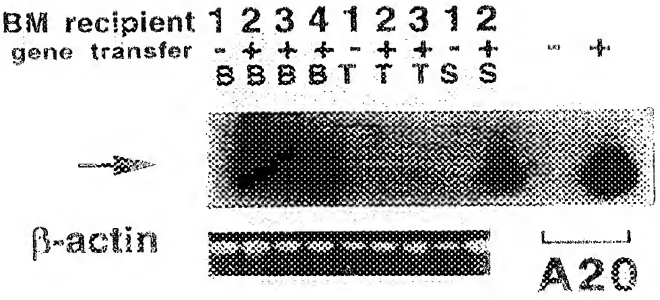

BM recipient 1 2 3 4 1 2 3 1 2
gene transfer - + + + - + + - + - +
B B B B T T T S S
β-actin
A20

FIG. 15

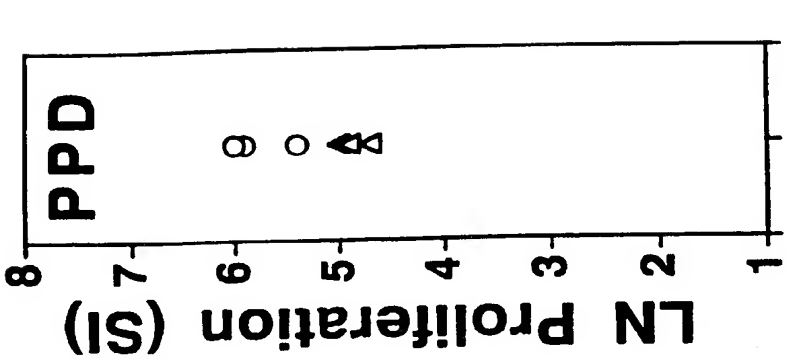


FIG. 16B

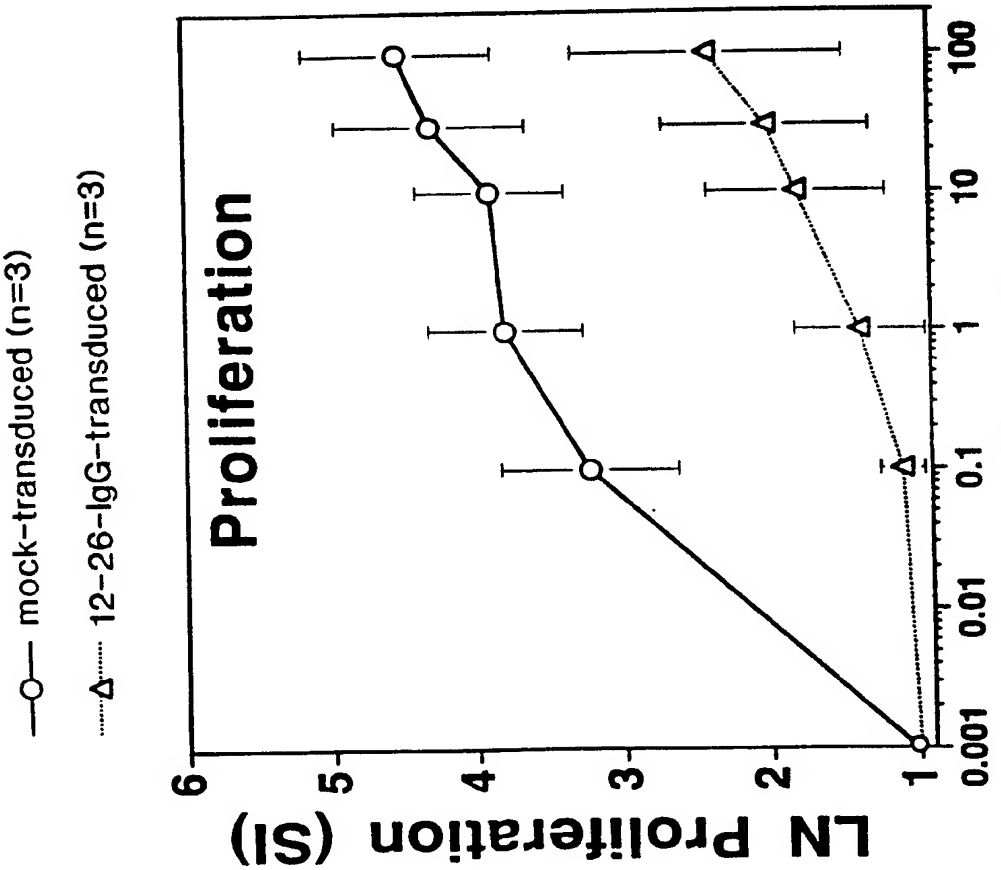


FIG. 16A

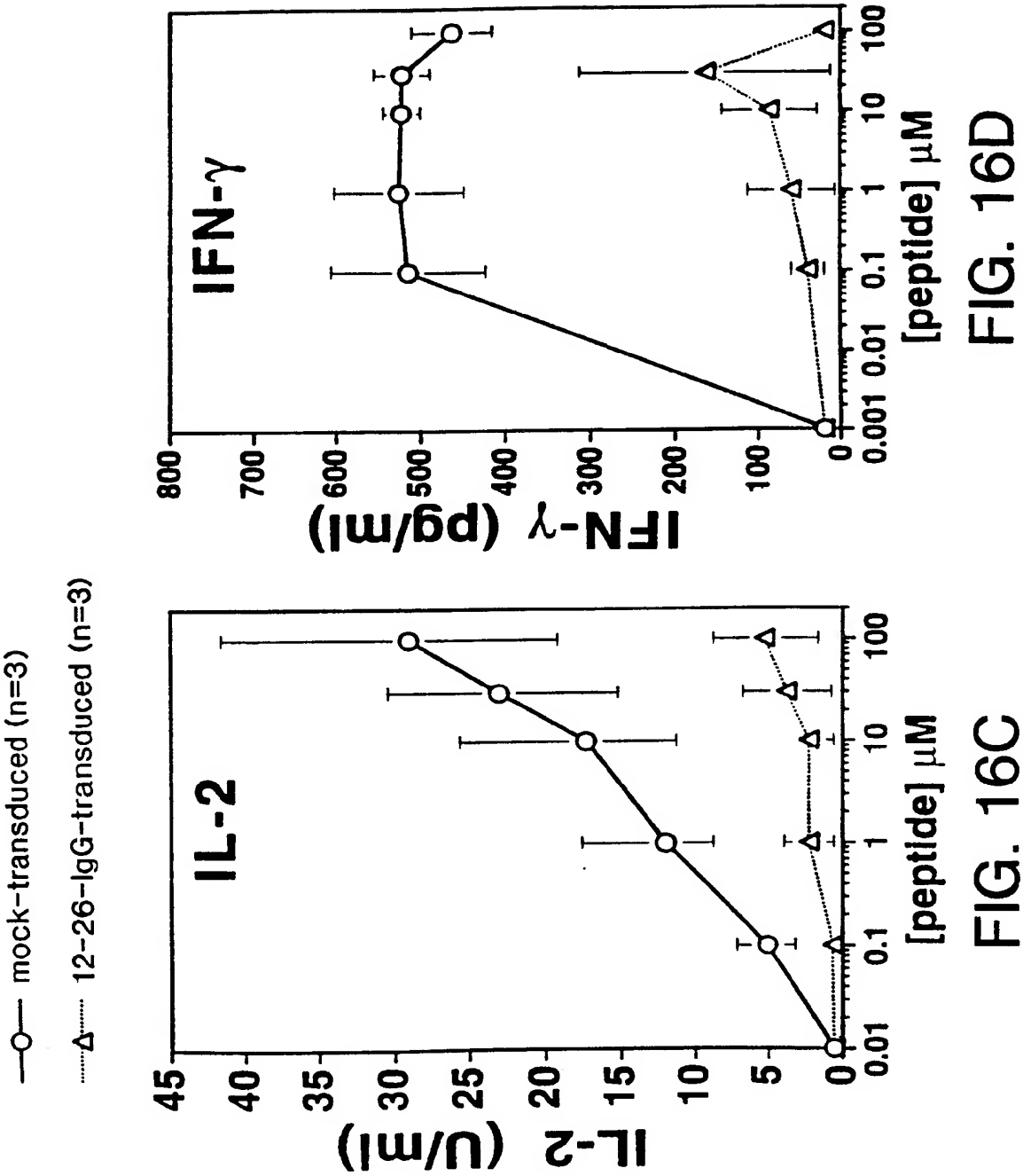


FIG. 16C

FIG. 16D

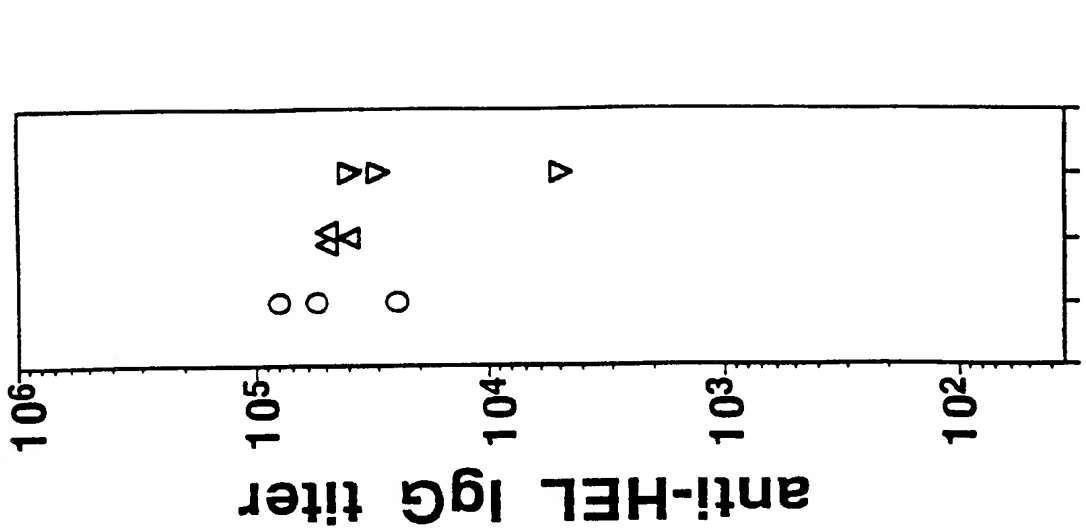


FIG. 17C

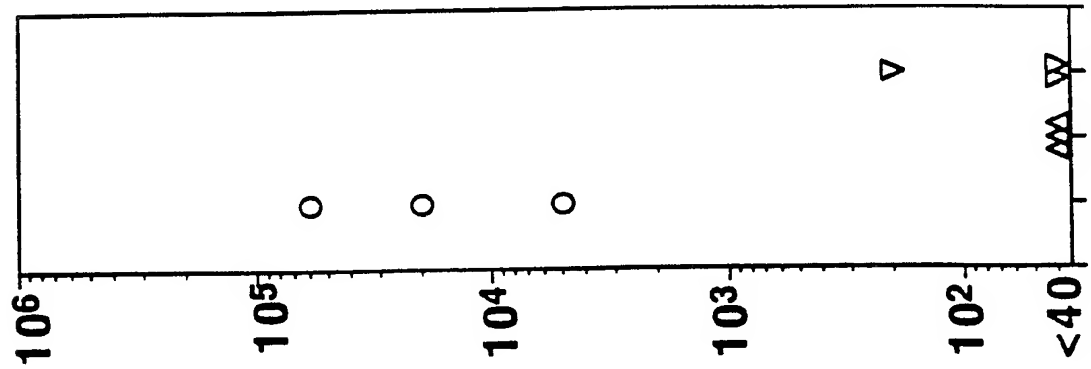


FIG. 17B

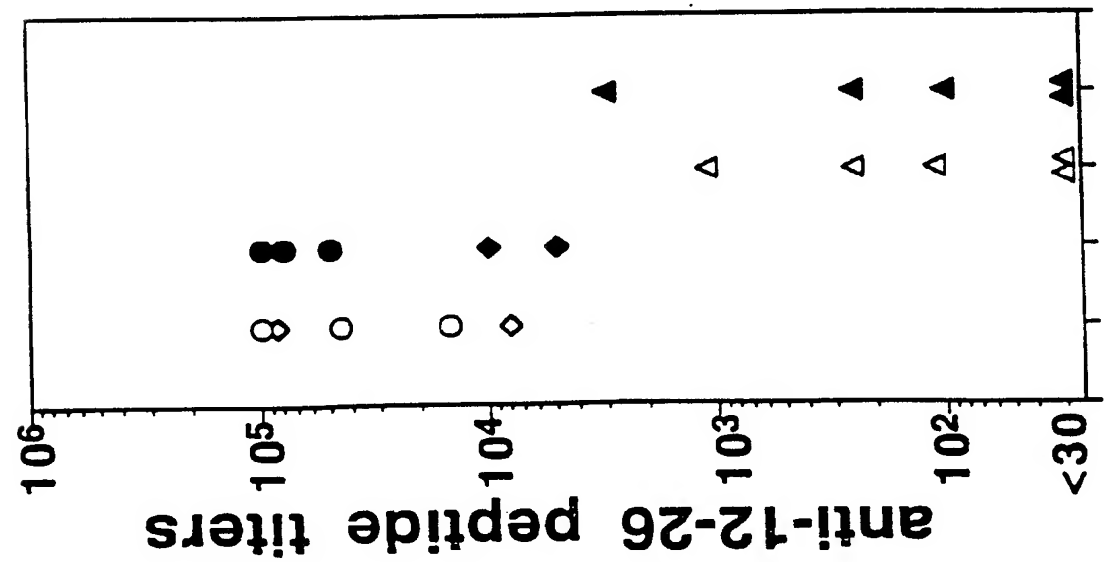


FIG. 17A

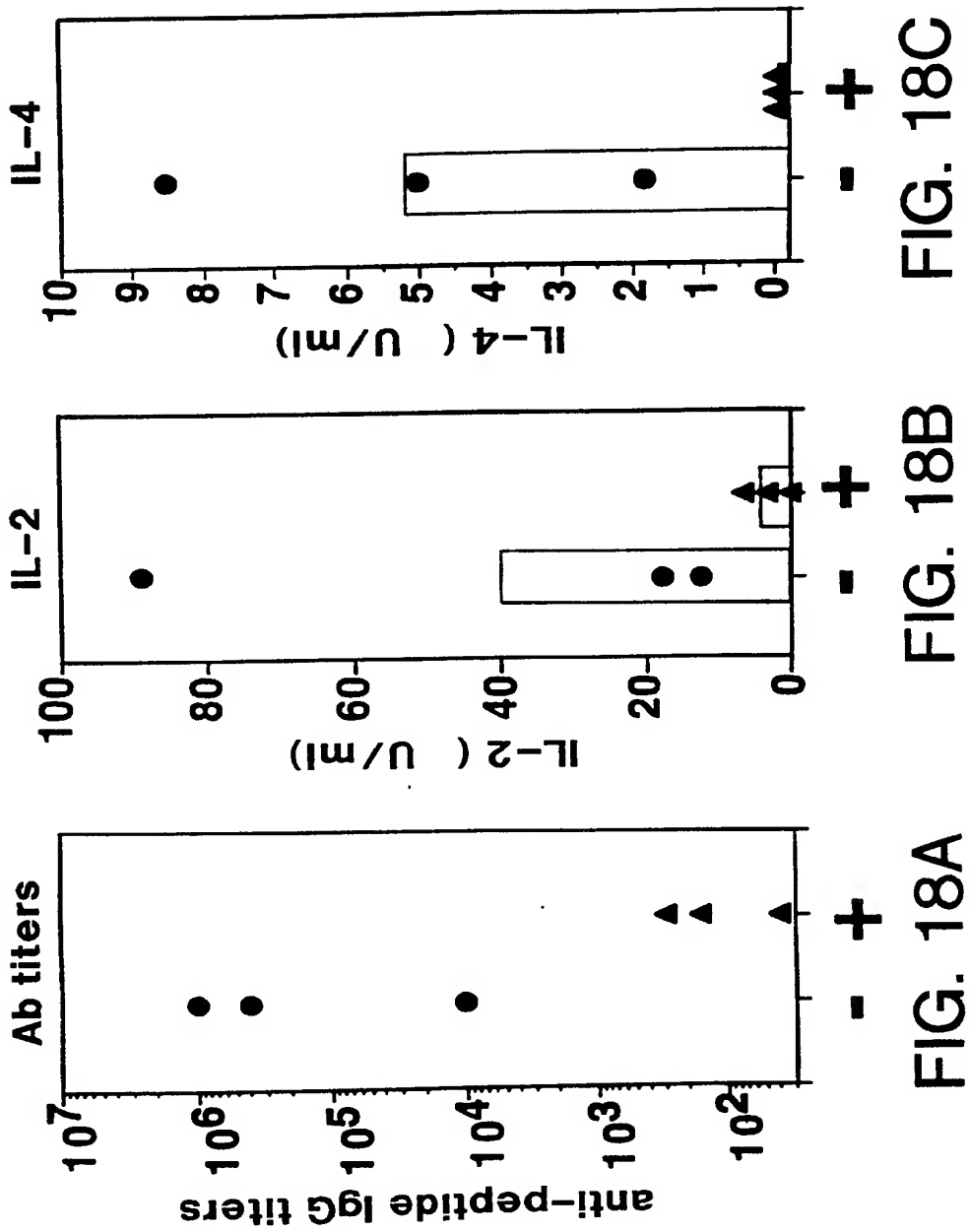


FIG. 18A

FIG. 18B

FIG. 18C

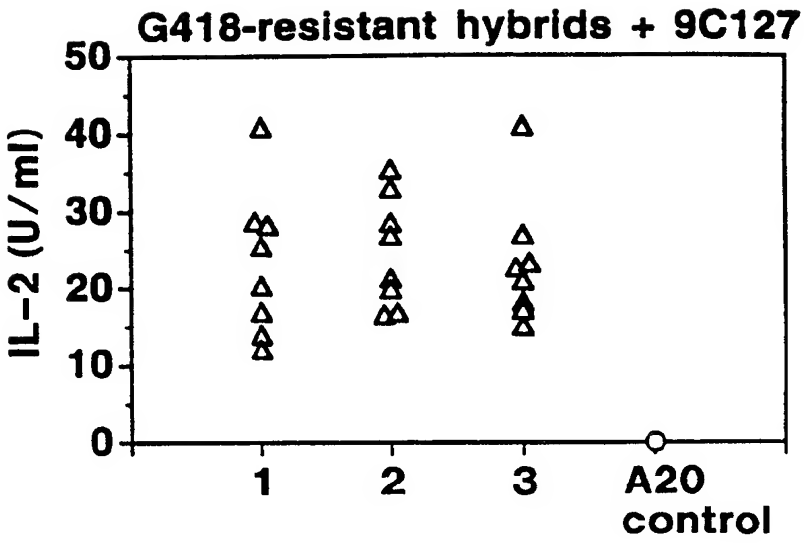

G418-resistant hybrids + 9C127
IL-2 (U/ml)
50
40
30
20
10
0
1
2
3
A20
control

FIG. 19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/02766

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/387.3, 388.35, 388.7; 536/23.4; 435/5, 7.1, 69.7, 326, 339.1; 424/139.1, 192.1, 208.1.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ZAMBIDIS et al., Epitope-specific tolerance induction with an engineered immunoglobulin, Proc. Natl. Acad. Sci. USA, May 1996, Vol. 93, pp. 5019-5024, especially abstract.	1-37
Y	FAITH, et al., Analysis of the basis of resistance and susceptibility of CD4+ T cells to human immunodeficiency virus (HIV)-gp120 induced anergy, Immunology, February 1992, Vol. 76, pp. 177-184, especially abstract, pp. 179-180.	1-37

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
24 APRIL 1998

Date of mailing of the international search report
18 JUN 1998

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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/02766

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HAYNES et al., Conversion of an Immunogenic Human Immunodeficiency Virus (HIV) Envelope Synthetic Peptide to a Tolergen in Chimpanzees by the Fusogenic Domain of HIV gp41 Envelope Protein, J. Exp Med, March 1993, Vol. 177, pp.717-727, especially abstract.	1-37

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/02766

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12P 21/04, 21/08; C07K 16/00; C07H 21/04; C12Q 1/70; G01N 33/53; A61K 39/00, 39/21, 39/395

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

530/387.3, 388.35, 388.7; 536/23.4; 435/5, 7.1, 69.7, 326, 339.1; 424/139.1, 192.1, 208.1.